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Determination of contaminants of emerging concern in surface water

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"One day, in retrospect, the years of struggle will strike you as the most beautiful." -
Sigmund **Freud**

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Abstract

Environmental pollution has increased due to the use of a wide range of organic compounds, resulting from anthropogenic activities. The uncontrolled discharge of such substances into the environment originates their accumulation in aquatic compartments, and might cause adverse ecological and human health effects, even when present at low concentrations. The grown concern about micropollutants in the environment and their pseudo-persistence trigger the need for development of trace methods for analysis of organic compounds in environmental matrices.

In this context, a sensitive multi-residue analytical method was developed and optimized based on solid-phase extraction (SPE) followed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) for surface water analysis of 11 contaminants of emerging concern (CECs), defined in the Watch List of European Commission Decision 2015/495/EU. The optimized mobile phase in a Kinetex™ 1.7 μm XB-C18 100 Å column was set as methanol/water (75:25, v/v) at gradient mode with a flow rate of 0.25 mL min^{-1}. The best recoveries for most target analytes were achieved with the Oasis® HLB cartridges, using ethanol as conditioning and eluting solvent and 500 mL of water samples at pH 3. The method was validated as recommended by international guidelines. The method detection limits were between 0.01 and 2.67 ng L^{-1} and the method quantification limits between 0.03 and 8.08 ng L^{-1}. The identification of the compounds was performed according to European Commission Decision 2002/657/EC, by analysing the retention time, two MS/MS transitions for each substance and its ion ratio.

The developed method was applied to 30 surface water samples collected in the spring of 2016, from the Sousa and Ave Rivers, both located in Northern Portugal. From the 11 studied compounds, 4 (diclofenac, 2-ethylhexyl 4-methoxycinnamate, clarithromycin and azithromycin) and 7 (diclofenac, 2-ethylhexyl 4-methoxycinnamate, erythromycin, clarithromycin, azithromycin, imidacloprid and thiamethoxam) were detected in Sousa and Ave River samples, respectively. The most frequently found micropollutants were clarithromycin and 2-ethylhexyl 4-methoxycinnamate in the Sousa River and azithromycin in the Ave River. The highest concentrations detected were for diclofenac (319.83 – 1855.95 ng L^{-1}) in the Sousa River and imidacloprid (up to 136.52 ng L^{-1}) in the Ave River. Other physical-chemical parameters were measured for both studied rivers.

Keywords: Contaminants of emerging concern; Commission Decision 2015/495/EU; surface water; solid-phase extraction; ultra-high performance liquid chromatography-tandem mass spectrometry; Sousa River; Ave River.

Resumo

A poluição ambiental tem vindo a aumentar devido ao uso de uma vasta gama de compostos orgânicos resultantes de atividades antropogénicas. A descarga descontrolada de tais substâncias no meio ambiente, ainda que em baixas concentrações, origina a acumulação destas substâncias nos compartimentos aquáticos e pode provocar efeitos adversos nos ecossistemas e na saúde humana. A crescente preocupação sobre a presença de micropoluentes e a respetiva persistência no meio ambiente desencadeou a necessidade de desenvolver métodos analíticos capazes de analisar compostos orgânicos em concentrações vestigiais (ng L^{-1} a µg L^{-1}) no meio ambiente.

Neste contexto foi desenvolvido e otimizado um método analítico com elevada sensibilidade para analisar, em águas superficiais, 11 contaminantes de preocupação emergente (CECs) definidos na lista de vigilância da Decisão da Comissão Europeia 2015/495/EU. O método envolveu extração em fase sólida (*SPE*) seguida de cromatografia liquida acoplada à espectrometria de massa em tandem (*LC-MS/MS*). A fase móvel foi otimizada numa coluna Kinetex™ 1,7 µm XB-C18 100 Å e consistiu em metanol/água (75/25, v/v) em modo gradiente com um caudal de 0,25 mL min^{-1}. Para a maioria dos compostos, as recuperações mais elevadas foram obtidas pela utilização de cartuchos Oasis® HLB e etanol como solvente de condicionamento e eluição, assim como um volume de 500 mL de amostra de água acidificada a pH 3. O método foi validado tal como recomendado pelas diretrizes internacionais. Os limites de deteção do método situaram-se entre 0,01 e 2,67 ng L^{-1} e os limites de quantificação entre 0,03 e 8,08 ng L^{-1}. A identificação dos compostos foi realizada de acordo com a Decisão da Comissão Europeia 2002/657/CE, através da análise dos tempos de retenção e da proporção entre as duas transições MS/MS para cada composto.

O método desenvolvido foi aplicado a 30 amostras de águas colhidas durante a primavera de 2016, nos rios Sousa e Ave, ambos localizados na região Norte de Portugal. Dos 11 compostos estudados, 4 (diclofenac, 4-metoxicinamato de 2-etil-hexilo, claritromicina e azitromicina) foram detetados no rio Sousa e 7 (diclofenac, 4-metoxicinamato de 2-etil-hexilo, eritromicina, claritromicina, azitromicina, imidaclopride e tiametoxame) no rio Ave. O diclofenac foi o composto determinado em concentrações mais elevadas no rio Sousa (319,83 – 1855,95 ng L^{-1}) e o imidaclopride no rio Ave (até 136,52 ng L^{-1}). Outros parâmetros físico-químicos foram analisados em ambos os rios.

Palavras-chave: Contaminantes de preocupação emergente; Decisão da Comissão Europeia 2015/495/UE; águas superficiais; extração em fase sólida; cromatografia líquida de ultra-alta eficiência; espectrometria de massa *em tandem*; rio Sousa; rio Ave.

Nomenclature

APCI – Atmospheric pressure chemical ionization

APPI – Atmospheric pressure photo ionization

BHT – 2,6-di-tert-butyl-4-methylphenol

CAS – Chemical Abstracts Service registry number

CEC – Contaminant of emerging concern

CI – Chemical ionization

CID – Collision induced dissociation

DAD – Diode array detection

DLLME – Dispersive liquid-liquid microextraction

DO – Dissolved oxygen

DOC – Dissolved organic carbon

DWTP – Drinking water treatment plant

E1 – Estrone

E2 – 17-beta-estradiol

EC – European Commission

EDC – Endocrine disrupting compound

EE2 – 17-alpha-ethinylestradiol

EHMC – 2-Ethylhexyl 4-methoxycinnamate

EI – Electron ionization

ESI – Electrospray ionization

EU – European Union

GC – Gas chromatography

GPS – Global positioning system

HLB – Hydrophilic–Lipophilic–Balanced

HPLC – High performance liquid chromatography

IC – Ion chromatography

IDL – Instrument detection limit

IL – Ionic liquid

IQL – Instrument quantification limit

LC – Liquid chromatography

LLE – Liquid–liquid extraction

LLME – Liquid-liquid microextraction

MAE – Microwave-assisted extraction

MAX – Mixed–mode anion exchange

MCX – Mixed–mode cation exchange

MDL – Method detection limit

ME – Matrix effect

MIP – Molecularly-imprinted polymer

MQL – Method quantification limit

MS – Mass spectrometry

MS/MS – Tandem mass spectrometry

MTBE – Methyl-tert-butyl-ether

Mw – Molecular weight

n.a. – not available

n.d. – not detected

NP – Nanoparticle

NSAID – Non-steroidal anti-inflammatory drug

PLE – Pressurized-liquid extraction

PPCP – Pharmaceuticals and personal care product

PS – Priority substance

PTFE – Polytetrafluoroethylene

QC – Quality control

QqQ – Triple quadrupole

QTOF/MS – Quadrupole-time of flight mass spectrometry

RSD – Relative standard deviation

SPE – Solid phase extraction

SPME – Solid phase microextraction

SRM – Selected reaction monitoring

TOC – Total organic carbon

TOF/MS – Time of flight mass spectrometry

UE – Ultrasonic extraction

UHPLC – Ultra–high-performance liquid chromatography

USA – Ultrasound-assisted

UV – Ultraviolet

WAX – Weak anion–exchange

WCX – Weak cation–exchange

WWTP – Wastewater treatment plant

Table of contents

Agradecimentos iii

Abstract v

Resumo vii

Nomenclature viii

1. General introduction 1

1.1. Contaminants of emerging concern 1

1.2. Water quality policy and European legislation 3

1.3. Analytical method development for determination of CECs in surface water 6

1.3.1. Extraction and concentration of target analytes: Solid Phase Extraction (SPE) 7

1.3.2. Detection of CECs 8

1.4. Case of study: Portuguese rivers 10

1.4.1. Sousa River 10

1.4.2. Ave River 11

1.5. Motivation and main objectives of this dissertation 12

2. State of the art 15

3. Analytical method development for determination of CECs in surface water 29

3.1. Chemicals and materials 29

3.2. Solid-phase extraction 34

3.3. Ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) parameters 35

3.4. Method parameters and validation 36

3.5. Matrix effect evaluation 38

3.6. Water sampling 38

3.7. Monitoring sites 39

3.7.1. Sousa River 39

3.7.2. Ave River 40

4. Results and discussion 41

4.1. UHPLC-MS/MS 41

4.1.1. Chromatographic separation 41

4.1.2. Mass spectrometry (MS / MS) 42

4.2. Solid-phase extraction optimization 44

4.2.1. Cartridges 44

4.2.2. Sample pH 45

4.2.3. Extraction solvent 45

4.2.4. Sample volume 46
4.3. Method validation 47
4.4. Matrix effects 51
4.5. Case of study: Sousa and Ave Rivers 51
4.5.1. Quantification of CECs 51
4.5.2. Physical-chemical parameters 54
4.5.3. Comparison of CECs in Sousa and Ave Rivers 61
5. Conclusions 63
6. Future work 64
References 645
Appendix A: Watch list of substances for Union-wide monitoring in the field of water policy 73
Appendix B: Mobile phase 74
Appendix C: MS parameters 75
Appendix D: Site pictures of the Sousa and Ave Rivers 78
Appendix E: Physical-chemical parameters 80
Appendix F: Concentration of CECs (ng L^{-1}) detected in Sousa and Ave River 84

List of figures

Figure 1. Schematic pathways of some CECs from sources to receptors (adapted from *[20]*). 2
Figure 2. Influence of polarity on chromatographic mass spectrometry technique as well as ionization conditions selection. Ion Source: ESI – Electrospray ionization; APCI – Atmospheric pressure chemical ionization; APPI – Atmospheric pressure photo ionization; EI – Electron ionization; CI – Chemical ionization. (adapted from [79]). 9
Figure 3. Triple quadrupole mass analyser. (adapted from [81]). 10
Figure 4. Sousa River (adapted from [83]). 11
Figure 5. Ave River (adapted from [83]). 12
Figure 6. Schematic representation of SPE procedure. 34
Figure 7. Manifold used to SPE procedure. 34
Figure 8. Equipment used to LC – MS/MS analysis. 36
Figure 9. Summary of sampling procedure. 39
Figure 10. Sampling points of Sousa River and respective GPS coordinates. 40
Figure 11. Sampling points of Ave River and respective GPS coordinates. 40
Figure 12. Combination of organic and aqueous phases tested. 41
Figure 13. Recoveries obtained for Watch List compounds for different cartridges (Oasis® HLB, MAX and MCX) extracting 250 mL of surface water samples (pH 3 for HLB and MAX; pH 11 for MCX) and using methanol as solvent. 44
Figure 14. Recoveries obtained for Watch List compounds for different pH (3, 7 and 11), extracting 250 mL of surface water samples through Oasis® HLB cartridges and using methanol as solvent. 45
Figure 15. Recoveries obtained for Watch List compounds for different solvents (ethanol, methanol and acetonitrile), extracting 250 mL of surface water samples through Oasis® HLB cartridges. 46
Figure 16. Recoveries obtained for Watch List compounds, extracting different sample volumes (100, 250, 500 e 1000 mL), of surface water samples (pH 3) through Oasis® HLB cartridges, using ethanol as solvent. 47
Figure 17. Flow rate (m^3s^{-1}) determined in the Sousa River. 52
Figure 18. Flow rate (m^3s^{-1}) determined in the Ave River. 52
Figure 19. Variation of concentrations (ng L^{-1}) of Watch List compounds found in Sousa River (data points with × correspond to < MQL values). 53
Figure 20. Variation of concentrations (ng L^{-1}) of Watch List compounds found in Ave River (data points with × correspond to < MQL values). 53
Figure 21. pH values and DOC concentrations (mg L^{-1}) in Sousa River. 55
Figure 22. Temperature values and DO concentrations (mg L^{-1}) in Sousa River. 56
Figure 23. Sodium, potassium, calcium and magnesium in Sousa River. 57
Figure 24. Chloride, nitrite, bromide, nitrate and sulfate concentrations (mg L^{-1}) in Sousa River. 57
Figure 25. pH values and DOC concentrations (mg L^{-1}) in Ave River. 58
Figure 26. Temperature values and DO concentrations (mg L^{-1}) in Ave River. 59
Figure 27. Sodium, potassium, calcium and magnesium concentrations (mg L^{-1}) in Ave River. 60

Figure 28. Chloride, bromide, nitrate and sulfate concentrations (mg L^{-1}) in Ave River. 60
Figure 29. Range of concentrations (ng L^{-1}) of CECS found in Sousa River. 61
Figure 30. Range of concentrations (ng L^{-1}) of CECS found in Ave River. 61
Figure B1. Chromatogram of the target analytes obtained with optimized mobile phase. Conditions: Kinetex™ 1.7 µm XB-C18 100 Å column (100 × 2.1 mm, i.d.), using a mobile phase of methanol/water (75/25, v/v) performed at gradient mode at a flow rate of 0.25 mL min^{-1} 74
Figure C1. Results obtained for target analytes with different nebulizing gas flow values: 1.0, 1.5, 2.0, 2.5 and 3.0 dm^3 min^{-1}. 75
Figure C2. Results obtained for target analytes with different drying gas flow values: 10.0, 12.5 and 15.0 dm^3 min^{-1}. 75
Figure C3. Results obtained for target analytes with different capillary voltage values: 1.5, 2.5, 3.5 and 4.5 kV. 76
Figure C4. Results obtained for target analytes with different desolvation temperature values: 200, 225, 250 and 300 ºC. 76
Figure C5. Results obtained for target analytes with different source temperature values: 200, 300, 350, 400 and 450 ºC. 77
Figure D1. Site pictures of the Sousa River. 78
Figure D2. Site pictures of the Ave River. 79

List of tables

Table 1. Monitorization studies of Watch List compounds in surface water. Other compounds, not included in the Watch List, are not addressed. 16
Table 2. Watch List compounds: group, CAS Number, molecular weight (Mw), structure, solubility and pKa. 30
Table 3. Optimized gradient mode. 42
Table 4. Selected reaction monitoring (SRM) instrument parameters for tandem mass spectrometry analysis of target analytes. 43
Table 5. Retention time, range, linearity, instrument and method detection and quantification limits for each target analyte 48
Table 6. Recovery, accuracy, precision (intra- and inter-batch) and matrix effect for each target analyte. 50
Table A1. Watch list of substances for Union-wide monitoring in the field of water policy defined in the Commission Implementing Decision 2015/495/EU of March 2015 [29]. 73
Table E1. Physical-chemical parameters measured in Sousa River. 80
Table E2. Ions concentration measured in Sousa River (mg L^{-1}). 81
Table E3. Physical-chemical parameters measured in Ave River. 82
Table E4. Ions concentration measured in Ave River (mg L^{-1}). 83
Table F1. Concentration of CECs (ng L^{-1}) detected in Sousa and Ave River samples analysed. 84

1. General introduction

1.1. Contaminants of emerging concern

Water is the main natural resource for humans, being also indispensable for all ecosystems. The importance and the limited availability of this resource has promoted the increase of its economic, environmental and social value. Today, the world faces an environmental worldwide crisis, as result of anthropogenic pressure and increased water consumption, along with its waste. This situation might be worsened in the near future, due to the climate changes and the effects of the asymmetric water distribution in the world [1]. As result of anthropogenic activities, environmental pollution has increased due to the use of a wide range of organic compounds. Ubiquitous sources of production, use and disposal of numerous chemicals commonly used in medicine, industry, agriculture and even common household conveniences [2] led to the widespread occurrence of micropollutants [3]. The uncontrolled discharge of such substances into the environment originates their accumulation in aquatic compartments, the final receptacle of diffuse pollution, with potentially detrimental effect to aquatic ecosystems and to human health [4, 5]. Recent research suggests that even low concentrations of micropollutants in the environment (ng L^{-1} to μg L^{-1}), might originate adverse ecological and human health effects [6].

A grown concern about micropollutants due to their presence and ability to pseudo-persist in the environment, namely in fresh water resources, arouse the technological advances of sensitive analytical instrumentation and the development of trace methods of analysis of organic compounds [7]. The presence of some drugs, pesticides, personal care products, among others, in groundwater, river water, sediments, soils and oceans, was already reviewed two decades ago [8, 9] and thousands of works have been reported until today.

Nowadays, an increasing interest raised about the fate and effects of the so-called contaminants of emerging concern (CECs) [10], which are organic micropollutants with a wide range of chemical nature [11, 12]. CECs are unregulated pollutants, which can be found in the environment at trace concentrations (ng L^{-1} to μg L^{-1}), with negative impact on water quality [9]. CECs are not restrict to newly developed compounds, but include various compounds used in everyday life. Their continuous introduction into environmental compartments turns them pseudo-persistent, increasing the potential to trigger off harmful effects. CECs belong to important chemical contaminants currently found in the environment [13, 14], comprising three large groups of compounds [14]: (i) substances that have been introduced into the environment recently, such as industrial compounds that have only recently been synthetized; (ii) compounds known for a longer time as present in the environment (e.g., hormones), but only recently recognized as potentially dangerous to ecosystems and/or humans [15]; and (iii) compounds that were only recently

detected despite being present in the environment for a long time. Actually, some of them come from industrial, medicinal and household usage, runoff from agriculture, livestock and aquaculture [16], being released worldwide into environmental compartments for decades [17, 18]. Recent studies corroborate the occurrence of CECs in surface, ground and tap water [9]. In fact, the environmental analysis of CECs is a complex challenge for researchers, due to the complexity of matrices, the diversity of chemical properties of the analytes and their very low concentrations. Nevertheless, the novel technologies developed in the last decade and the increased sensitivity of the available analytical instruments has extended the spectrum of compounds that can be determined [19]. Figure 1 shows a general example of the sources, pathways and receptacles of CECs in the environment.

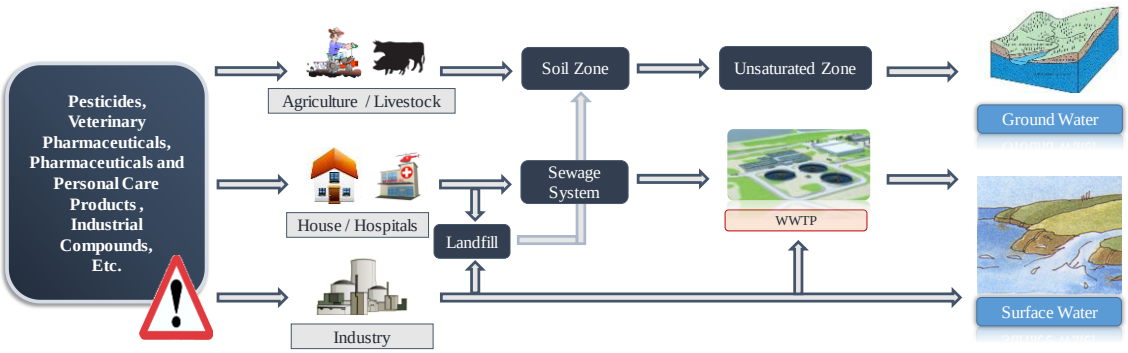


Figure 1. Schematic pathways of some CECs from sources to receptors (adapted from *[20]*).

Regarding occurrence of pharmaceuticals and hormones, they can be metabolized and excreted (unchanged or as metabolites), achieving the wastewater treatment plants (WWTPs) through the sewage network [10]. On the other hand, direct release may occur by improper dump of unused or expired drugs directly in toilet sinks or as solid waste. Veterinary pharmaceuticals are used for pets, livestock and aquaculture, which excrete drugs and metabolites [21]. Another important source of micropollutants, particularly in the case of pesticides, insecticides and herbicides, is agriculture where these CECs are used to protect plants and improve productivity [22, 23]. In addition, industrial compounds, many of them without regulations in some regions of the world, achieve directly surface water [9] leading to several risks to the environment. However, even in regulated countries, where some industrial effluents are discharged into conventional WWTPs, the CECs will not be removed completely [17]. It is consensual among the scientific community, that the main pathway of micropollutants into the aquatic environment is the release from WWTPs through treated effluents from domestic and/or industrial activities, as well as from hospitals [9]. Conventional WWTPs are not originally designed for elimination of potential toxic compounds and their efficiency to remove this type of compounds is not yet clearly understood [11].

The presence of micropollutants in aquatic environment has particular concern, because native organisms are subjected to exposure with potential consequences for future generations [24]. The problematic of CECs is the lack of knowledge about the middle and/or long-term effects to ecosystems and human health [17]. Therefore, the precautionary behaviour should be kept in mind due to chronic and long-term exposure [17]. Their continuous but non detected effects may gradually accumulate, leading to irreversible changes on both wildlife and human health [25]. Aquatic species have a major risk of exposure to individual agents or combinations of these compounds [17]; however, humans also depend on the fate and behaviour of CECs in surface water used to supply the drinking water treatment plants (DWTPs). Since the presence of these compounds in surface and ground water is normally found at trace concentrations, the acute toxicity is less likely to occur but easier to evaluate than chronic toxicity resulting from long-term exposure.

1.2. Water quality policy and European legislation

As referred above, occurrence of micropollutants in the aquatic environment and their effects to ecosystems and humans is a recent challenge. Water quality is one of the priority issues of environmental policy agenda considering the increasing demand for safe water.

Some European regulations have been published since the year of 2000, when the Directive 2000/60/EC was launched to establish a framework for the Community action in the field of water policy [26]. This mark represented a huge improvement in water protection policy, with the aim of achieving good ecological and chemical status of surface water. More recently, Directive 2008/105/EC [27] amended the above mentioned Directive and set out the first list of 33 priority substances/group of substances (PSs) that should be monitored for action at Community level. Five years later, the Directive 39/2013/EU [28] amended the previous documents, recommending the monitoring of 45 PSs and highlighting the demand to develop new water treatment solutions. This Directive proposed a first Watch List of substances for Union-wide monitoring in the field of water policy, which was then published in the Decision 2015/495/EU of 20 March 2015 [29].

The Watch List of Decision 2015/495/EU [29] (Appendix A – Table A.1) contemplates 10 substances/group of substances for which Union-wide monitoring data need to be gathered for the purpose of supporting future prioritisation exercises. This also includes an indication of the matrices to be monitored and possible methods of analysis for each substance/group of substances [29]. Two natural hormones (17-beta-estradiol - E2 and estrone - E1), a synthetic hormone (17-alpha-ethinylestradiol - EE2), a non-steroidal anti-inflammatory drug (NSAID) (diclofenac), three macrolide antibiotics (azithromycin, clarithromycin and erythromycin), two herbicides (oxadiazon and triallat), five neonicotinoid pesticides (imidacloprid, thiacloprid, thiamethoxam,

clothianidin and acetamiprid), a pesticide (methiocarb), an UV filter and an antioxidant commonly used as food additive are the specific 17 compounds included in the list of 10 substances/group of substances, referred in the Watch List [9, 29], which are briefly described below.

- **Hormones (17-beta-estradiol, estrone and 17-alpha-ethinylestradiol)**

Since three decades ago, it has been shown that specific synthetic and/or natural chemicals in the environment can disturb the normal endocrine system function of exposed organisms by interfering with the action of hormones [30, 31]. 17-beta-estradiol, estrone and 17-alpha-ethinylestradiol are biologically active substances known as endocrine disrupting compounds (EDCs). The United States Environmental Protection Agency describes an EDC as: "an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development, and/or behaviour" [32]. The hormones referred in the Watch List may pose a threat to the environment with a range of possible adverse effects on ecosystems and human health including chronic and acute toxicity to organisms, accumulation in ecosystems and loss of biodiversity [33, 34].

- **Pharmaceuticals: NSAID (diclofenac) and antibiotics (erythromycin, clarithromycin and azithromycin)**

A medicinal product for human use is defined by Directive 2001/83/EC and amended by Directive 2004/27/EC as "any substance or combination of substances presenting properties for treating or preventing disease in human beings or which may be used in or administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action" [35]. Considering that pharmaceuticals are specifically produced to change biochemical and physiological functions and many of them might remain active in the environment, acting on non-target organisms, this class of compounds is one of the largest of CECs [36]. Consequently, their release into the environment poses a hazardous problem and sub-lethal effects can occur [25, 37] as example, the development of resistance by aquatic microorganisms, adverse effects on nitrifying bacteria or growth inhibition of crop plants and weeds by bioaccumulation and inhibition of algal reproduction [38]. Still, the concrete effects in the organisms mainly depend on the chemical nature, concentration, and other factors, as adsorption coefficients, exposure time and bioaccumulation [39]. Diclofenac is a NSAID frequently detected in effluents, surface water and even ground and tap water. Even at trace concentrations, it is known to harmfully affect several species [40]. On the other hand, a particular importance has been given to antibiotics, due to their potential to originate the development of

resistant mechanisms by bacteria. Macrolide antibiotics, such as azithromycin, clarithromycin and erythromycin are broadly used in human and veterinary medicine, as well as in aquaculture, for the purpose of preventing or treating serious infections induced by *pneumococci, staphylococci and streptococci* [41, 42].

- **Food Additive (2,6-di-tert-butyl-4-methylphenol)**

2,6-di-tert-butyl-4-methylphenol (BHT) exerts efficient antioxidant activity and has been applied to conserve and stabilize the nutritive value, flavour and colour of food and animal feed products, since the middle of the 20th century, being authorized in approximately 40 countries as a direct or indirect food additive [43]. Although the use of BHT as food antioxidant apparently does not show a public health risk to the environment, this compound is degraded biologically to 3,5-di-tert-butyl-4-hydroxybenzaldehyde, known by generating peroxides in rats and inducing cellular DNA damage [44].

- **Organic UV Filter (2-ethylhexyl 4-methoxycinnamate)**

2-Ethylhexyl 4-methoxycinnamate (EHMC) is an organic substance used as UV filter or UV light stabilizer to avoid photo-deterioration of human skin or plastic products, respectively [45]. Its potential to cause hormonal alterations that have been reported *in vitro* as well as *in vivo,* raised environmental concerns [46, 47]. As example, Zucchi et al. (2010) showed that EHMC induced a decrease of both spermatocytes in tests and previtellogenic oocytes in ovaries of zebra fish [48]. Other observations showed that EHMC was involved in coral bleaching by supporting viral infections [49].

- **Pesticides (Methiocarb)**

Methiocarb is one of the most frequently used carbamate pesticides worldwide [9]. Since the detection of pesticides in the environment in the 1960s, the concern related to water supplies has increased [50]. Pesticides embrace a large number of substances with common characteristics such as the effectiveness against pests. Contamination of surface and ground waters with agricultural pesticides remains a problem even when they were phased out due to their extensive use for a large number of years [51]. Pesticides and their degradation products are very toxic and very resistant and remain in the Nature for many years [52], thereby their impact in the environment and wildlife includes the enhancement of the incidence of cancer, genetic mutations and other diseases affecting mainly liver or the central nervous system [9, 53].

- **Neonicotinoids (Insecticides – imidacloprid, thiacloprid, thiamethoxam, clothianidin and acetamiprid)**

Neonicotinoids (neonics) were synthesized in 1970s for the first time, and Bayer patented imidacloprid as the first commercial neonicotinoid in 1985 [54]. Nowadays, neonics such as imidacloprid, thiacloprid, thiamethoxam, clothianidin and acetamiprid, are used widely to treat crops (corn, soybean, barley, oat, etc.) and to protect livestock animals against insects [55, 56]. The high selective affinity to insects makes neonicotinoids not expected to be highly toxic compounds to non-target organisms, such as mammals, and theoretically they are safer for the environment than other biocides [55]. However, the wide use of neonicotinoids and recent research brought an increasing concern, suggesting that these compounds could induce liver tumours, oxidative stress and inflammation in the central nervous system. In addition, these compounds may cause an impact on the structure and functionality of aquatic invertebrate communities and contribute to decline of honey bee colonies and insectivorous birds population [57, 58], even at $\mu g\ L^{-1}$ levels.

- **Herbicide (oxadiazon e triallat)**

The large use of plant protection products and their recurrent intentional distribution on arable land led to the contamination of groundwater and surface water [59]. Various herbicides including oxadiazon were already identified as inducers of toxic effects on primary producers [60]. Although oxadiazon and triallat are widely used to control annual and perennial grasses in wheat, legumes and a large number of other crops, they are organic contaminants of high environmental concern due to their relatively long half-life [9, 61]. Many studies on environmental fate and toxicological properties of such compounds are still needed to evaluate their ‘‘emerging’’ status depending on their persistency and toxicity for humans and ecosystems [13].

1.3. Analytical method development for determination of CECs in surface water

The development of robust analytical methods to determine CECs in the environment has been a main challenge for analytical chemistry researchers for the last 20 years [62]. The development of analytical methodologies for the determination of CECs has been mainly focused on the evaluation of the occurrence of products used continuously on daily basis, like health care and agriculture products, among others [63]. Several reports describe the determination of CECs in surface water, many of which are multi-residue methods [64-66], but none is focused on the CECs listed in the Watch List. The complexity of environmental matrices and the low concentrations

detected in the environment, namely in surface water, demand sample pre-concentration and removal of the interferences present in these samples, before chromatographic analysis [51]. The progress of extraction techniques, increasingly simple and inexpensive, provides the enhancement of target analyte recoveries from environmental matrices [67].

Decision 2015/495/EU [29] suggests the methods of analysis for each CEC included in the Watch List. Solid-phase extraction (SPE) coupled to liquid chromatography tandem triple quadrupole mass spectrometry (LC–MS/MS) is indicated for most compounds [29]. In fact, LC-MS/MS allows the routine analysis of all kinds of non-volatile polar organic compounds, which could not be detected before without derivatization using gas chromatographic approaches, early extensively used for detection of many persistent and non-polar organic pollutants [33, 34]. Nevertheless, gas chromatography mass spectrometry (GC-MS/MS), which is out of the scope of the present report, is preferential for some CECs, such as, 2-ethylhexyl 4-methoxycinnamate, methiocarb, oxadiazon and triallat [29].

1.3.1. Extraction and concentration of target analytes: Solid Phase Extraction (SPE)

Normally, two thirds of the analysis time is spent on collection and preparation of the samples [68]. Therefore, these steps are a critical hindrance in all over analytical process [69]. Errors related to the handling steps should be reduced and an accurate and precise sample preparation is crucial for analysis of CECs [70]. Once CECs are highly diluted in surface water samples and large amounts of interferences can be present, sample preparation techniques allow achieving the extraction of target analytes at such residual concentrations and simultaneously permit the removal of other non-target compounds that probably would reduce the efficiency of analysis [69]. Nowadays, different techniques for sample extraction/concentration are applied, however SPE is the most used technique to pre-treat surface water samples [68].

SPE is a sample preparation technique, used from the late of 70s for concentration of pollutants in water samples, with proved advantages over other techniques, including low organic solvent consumption, simplicity, celerity and great sample clean-up. It is a very popular technique in different research areas such as environmental, clinical, biological and food fields [71]. The main steps of SPE procedure are: i) adsorbent conditioning, ii) sample loading, iii) washing, iv) dryness, v) elution, vi) evaporation of the organic phase, and vii) reconstitution of the extract to analyse [70].

Apart from the extraction and concentration of target analytes, as well as the removal of interferences, other recent goals of the SPE procedure have been set, including the reduction of initial volume sample, improvement of selectivity in extraction and minimizing the amount

organic solvents, of more relevance when they are not eco-friendly [69, 72]. This technique has also some disadvantages, such as the multi-steps needed, time consuming, influence of the operator, losses in the evaporation step, risk of contamination, the clogging of cartridges and low recoveries in some cases [70, 73].

Several SPE sorbents have been developed and their choice is a conditioning factor to achieve the optimal conditions, since it will affect directly the selectivity and affinity of target analytes, affecting the SPE yield. The different interactions between sorbent and analytes, such as hydrophobic/hydrophilic interactions and cation/anion exchange processes, should be taken into account [73]. Different types of solid-phase sorbents, such as hydrophilic-lipophilic balance (e.g., Oasis® HLB), ion-exchange (e.g., weak anion–exchange, Oasis WAX; weak cation–exchange, Oasis® WCX) mixed-mode/ionic-exchange (e.g., mixed-mode/anion-exchange, Oasis® MAX; mixed-mode/cation-exchange, Oasis MCX) are frequently used for SPE. However, a large number of new sorbents for SPE has been suggested and applied for sample preparation, such as magnetic nanomaterials, molecularly-imprinted polymers (MIPs) and carbon nanoparticles (NPs) [69]. Other important parameters affecting the efficiency of the extraction process include the solvents used, sample volume load, pH, use of additives to increase the extraction, etc. [74].

Among other extraction techniques, conventional liquid-liquid extraction (LLE) is a classic method also frequently employed; however, it has numerous disadvantages, such as emulsion formation, use of large volumes of solvents, most of them toxic, time consumption and several steps involved [73]. The requirement for eco-friendly procedures has resulted in attempts to adapt traditional LLE methods into dispersive liquid-liquid microextraction (DLLME), which employs a smaller volume of organic solvent [71, 75]. Solid phase microextraction (SPME) is similar to SPE but has the advantage of using smaller volume of solvent and the extraction is performed based on one step equilibrium of target analytes instead of multi-step equilibrium in SPE, but have been more employed in GC procedure [71]. More recently, new extraction techniques have been applied with good results, such as ultrasonic extraction (UE), pressurized-liquid extraction (PLE) and microwave-assisted extraction (MAE) [73, 76].

1.3.2. Detection of CECs

After extraction and concentration of CECs, chromatographic methods are normally used to identify and quantify numerous compounds in a single analysis. These methods should be sensitive enough to detect trace levels [77]. The presence of micropollutants at residual levels in environmental aquatic samples and the high complexity of the matrix, such as surface water, require the use of selective and sensitive analytical techniques. In this sense, GC-MS/MS and LC-MS/MS are the main techniques recommended by Decision 2015/495/EU to analyse the Watch

List CECs [29]. The election of the separation technique is conditioned by the characteristics of the compounds, such as volatility and polarity. The volatile, semi-volatile and thermally stable compounds should be determined by GC; however, polar, non-volatile and/or thermally unstable ones should be determined by LC. Figure 2 shows the influence of the analyte polarity on the selection of chromatographic mass spectrometry (MS) technique as well as ionization conditions [78].

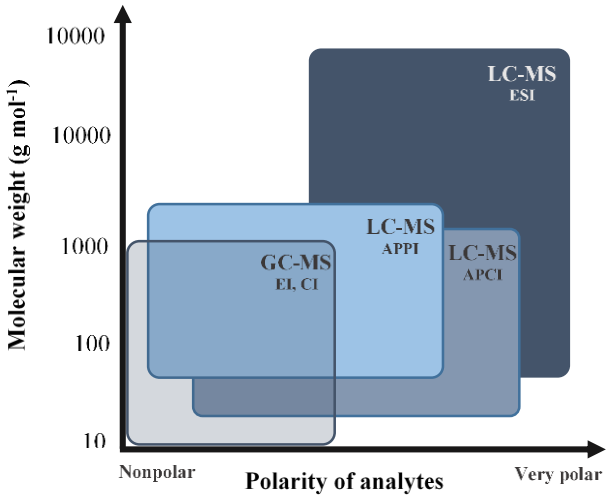


Figure 2. Influence of polarity on selection of chromatographic MS technique as well as ionization conditions. Ion Source: ESI – Electrospray ionization; APCI – Atmospheric pressure chemical ionization; APPI – Atmospheric pressure photo ionization; EI – Electron ionization; CI – Chemical ionization. (adapted from [79]).

LC-MS/MS is the most usual technique for determination of CECs in water matrices. This technique provides information about the structure of the analytes, without the need of derivatization, enabling a simultaneous analysis of a wide range of substances [80]. MS is an analytical technique that provides the identification and quantification of organic compounds, through the measure of the mass-to-charge ratio (m/z) of charged molecules. MS consists of three basic components: an ion source, an analyser where ions are separated according to their m/z and a detector where ions are counted. The analyser and detector of a mass spectrometer are kept in a high vacuum to avoid unintentional collisions with air molecules [80]. Coupled to liquid chromatograph, Decision 2015/495/EU suggests triple quadrupole (QqQ) tandem mass spectrometry as mass analyser (Figure 3) [29]. In fact, QqQ is currently the most popular detector used in combination with a liquid chromatograph in the analysis of micropollutants, where two quadrupoles (Q1 and Q3) act as ion analysers and the other quadrupole (Q2, referred as q in QqQ nomenclature) operates as a collision cell [80].

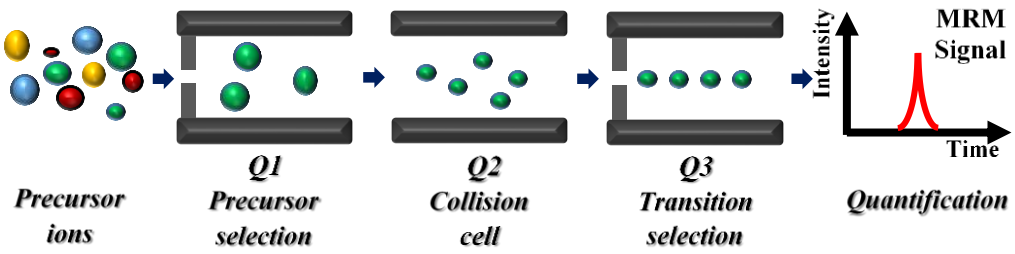


Figure 3. Triple quadrupole mass analyser. (adapted from [81]).

Precursor ions, with a specific m/z value, pass through the first quadrupole (Q1). In the second one (Q2), these precursor ions are subjected to fragmentation in the collision cell filled with an inert gas. The fragments are selected by the third quadrupole (Q3) and the fragmentation spectrum, i.e., the m/z values of the transitions between precursor and products, are registered by the detector. This type of working mode of the spectrometer is the so called multiple reaction monitoring mode (MRM) [80].

1.4. Case of study: Portuguese rivers

The selection of the Sousa and Ave Rivers was based on their recognized pollution levels and also due to the distance between the institution where this work was developed (FEUP, Porto) and such rivers, both located in Northern Portugal.

1.4.1. Sousa River

Sousa River (Figure 4) is a right bank tributary of Douro River. It rises in Friande (Felgueiras), runs through 64.7 km until its mouth in Foz do Sousa (Gondomar). The main tributaries are Cavalum, Mesio and Ferreira Rivers. The last one has a great significance and intersects the Sousa River near Foz do Sousa (Gondomar) [82].

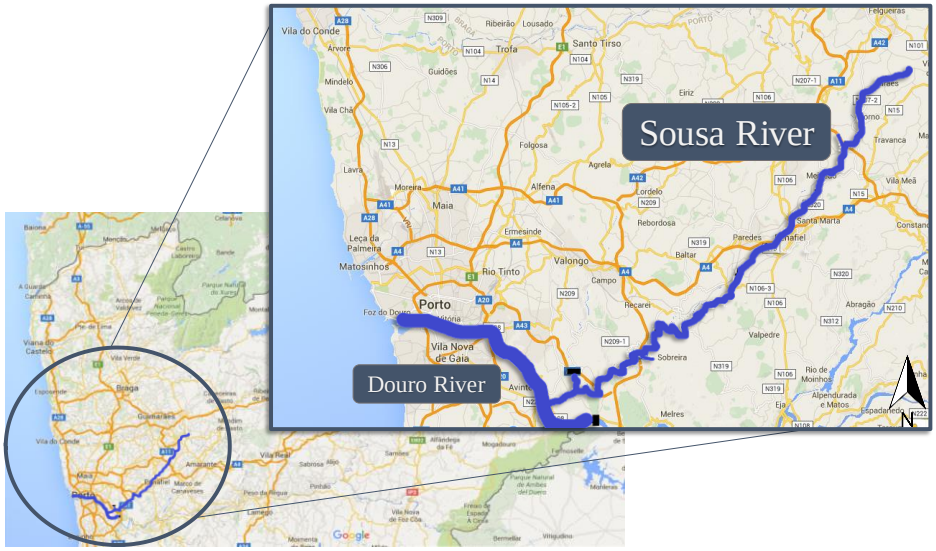


Figure 4. Sousa River (adapted from [83]).

Along its path, this river crosses some cities such as Felgueiras, Lousada, Penafiel, Paredes and Gondomar [82]. Paredes and Penafiel represent approximately 50% of the total agricultural production of cereals of the Porto Metropolitan Area. It is important to highlight the flower production in Paredes area. Concerning the livestock production of the Sousa Valley, cattle represents 70% of agricultural holdings, mainly in Paredes and Lousada [82]. There are two WWTPs on the margins of the Sousa River which effluents are drained to the river and some micro-hydro plants along the course. According to data, at least until 2009, the Sousa River was used as source of water to DWTPs. However, water quality monitoring has shown high levels of bacterial and organic contamination trending to increase over time as a result of high population density and intense industrial activity [82].

1.4.2. Ave River

Ave River (Figure 5) has an extension rounding 90 km, from its source located in Serra da Cabreira at 1260 m of altitude, to the mouth in Vila do Conde. This river receives water from a large set of rivers or brooks, including Cabreiro, Caniçado, Falperra, Vizela, Selho, Pele, Pelhe and Este. The Este River (right margin) and Vizela River (left margin) are its major tributaries [84].

Figure 5. Ave River (adapted from [83]).

The higher amount of livestock farms, as intensive regime, are located near Póvoa de Varzim, Vila do Conde and Vila Nova de Famalicão. In 1996, the Ave River was already considered a highly polluted river and the most industry surrounded river of Portugal. Even after the construction of some WWTPs, the Ave River still shows high levels of pollution [84]. Besides the chemical contamination, recent studies indicated the presence of 4 strains of resistant bacteria [85].

1.5. Motivation and main objectives of this dissertation

Regarding the knowledge about CECs in surface and ground waters, there is a huge amount of studies about their occurrence [9]. Most countries do not have appropriate legislation or monitoring programs to routinely determine micropollutants; however, there are many reports dealing with monitoring campaigns and development of methods to determine CECs. The state of the art (Section 2) of this document describes some works already published on development of analytical methods to assess the occurrence of CECs enlisted in the Watch List of Decision 2015/495/EU [29]. Literature on the fate of these compounds is extensive, but systematic studies on the occurrence of the majority of the referred CECs and rigorous data about their environmental effects at the trace concentrations at which they are usually found, are both limited [24].

As above mentioned for each group of compounds, in Section 1.2, there is a high interest in some categories of micropollutants, with particular chemical structures and properties, mainly interfering with the nervous central system, endocrine system and others. These environmental contaminants are poorly inventoried and regulated and insufficient information exists about their occurrence, fate and impact [86-88].

The aim of this dissertation was the development, optimization and validation of an analytical method based on off-line SPE followed by Ultra-High Performance Liquid Chromatography coupled to Mass Spectrometry (UHPLC-MS/MS) for the determination of Watch List compounds defined in Commission Decision 2015/495/EU. A spatial monitoring campaign of Watch List compounds was also performed in the two rivers referred above (Sousa and Ave).

2. State of the art

The state of the art (Table 1) of the present dissertation considers studies on occurrence of Watch List compounds in surface water, and also the analytical methods performed in such works. Sample preparation and chromatographic analysis are detailed. This search is focused on Scopus online database, by searching documents dated from 2010, whose keywords comprised "*name of micropollutant*" plus "*surface water*", where the name of micropollutant includes all the CECs enlisted in the Watch List of Decision 495/2015/EU [29], namely: estradiol, estrone, ethinylestradiol, diclofenac, azithromycin, clarithromycin, erythromycin, oxadiazon, triallat, imidacloprid, thiacloprid, thiamethoxam, clothianidin, acetamiprid, methiocarb, 2-ethylhexyl 4-methoxycinnamate and 2,6-di-tert-butyl-4-methylphenol. Generally, surface water samples are collected in dark glass bottles and refrigerated until sample preparation. Regarding the sample preparation strategy, the cartridges Oasis® HLB are the most frequently used to cleanup and extract organic compounds from surface water whereas LC-MS/MS is the technique per excellence to determine micropollutants in aquatic matrices; however, other techniques as HPLC-DAD and UHPLC coupled to Quadrupole-time of flight mass spectrometry (QTOF/MS) were applied. Most surface water analysed was collected from rivers, but some works determined CECs in lagoons and also in seawater.

Diclofenac is one of the most studied Watch List compounds and EHMC was only found in one study in China [89]. Regarding to hormones, studies were performed in Malaysia, South Korea and Brazil [90-92]. BHT was not included in this Section since no studies were found with the same objective of this work. This literature survey gives an insight of the lack of a single methodology able to determine simultaneously all (or at least most of the) compounds included in the Watch List.

Table 1. Monitoring studies of Watch List compounds in surface water. Other compounds, not included in the Watch List, are not addressed.

Watch List Compounds	Matrix Concentration Location (number of samples)	Sampling Procedure / Sample Preparation	Chromatographic Conditions	# Sampling Points Frequency of occurrence	Ref.
Diclofenac Erythromycin	Surface water 1.1 – 15.6 μg L^{-1} 0.58 – 22.6 μg L^{-1} South Africa (n = 25)	Amber glass bottles; Stored at 4 °C and fixed with H_2SO_4 (50 %) during transportation. **SPE** **Cartridges**: Supelclean™ LC-18 and Oasis® HLB (150 mg, 6 mL); **Conditioning:** 6 mL of *n*-hexane + 2 mL of acetone + 10 mL of methanol + 10 mL of distilled water; **Sample loading:** 500 mL; **Elution:** 5 x 1 mL methanol/acetone.	HPLC – DAD **Column type:** Agilent C_{18} (150 x 4.6 mm i.d., 5 μm particle size); **Mobile phase:** 0.02 M ammonium acetate/methanol or acidified ultra-pure water/methanol; **Flow rate:** 0.4 mL min^{-1}.	15 sampling points; Diclofenac: 100% Erythromycin: 88%	[93]
Erythromycin	River water 3.5 – 126 μg L^{-1} South China (n = 48)	Brown glass bottles; Stored at 5 ºC. **SPE** **Cartridges**: Oasis® HLB (500 mg, 6 mL); **Conditioning:** 6 mL of methanol + 6 mL of ultra-pure water + 6 mL of 10 mM Na_2EDTA buffer; **Sample loading:** n.a.; **Elution:** 3 x 2 mL of methanol.	HPLC – MS/MS **Column type:** ODS-P (250 mm x 4.6 mm i.d., 3.5 μm particle size); **Mobile phase:** Acetonitrile/formic acid 0.2% (v/v) at gradient mode; **Flow rate:** 0.4 mL min^{-1}.	16 sampling points; Erythromycin: 90%	[94, 95]

Table 1. (Continued)

Watch List Compounds	Matrix Concentration Location (number of samples)	Sampling Procedure / Sample Preparation	Chromatographic Conditions	# Sampling Points Frequency of occurrence	Ref.
2-ethylhexyl-4-methoxycinnamate	River water 0.4 – 500 ng L^{-1} China (n = 5)	Amber glass containers; Stored in the dark at 4 ºC. **<u>USA-IL-DLLME</u>** **Extraction:** 30 µL of ionic liquid + 100 µL of disperser solvent; **Ultrasonic water bath:** 35 kHz of ultrasound frequency + 320 W during 5 min at room temperature; **Centrifugation:** 4500 rpm during 5 min; **Sample loading:** 10 mL; **Elution:** 30 mL of methanol.	HPLC – DAD **Column type:** ZORBAX Eclipse XDB-C_{18} (150 mm x 4.6 mm i.d., 5 µm particle size); **Mobile phase:** methanol/water (90/10, v/v); **Flow rate:** n.a.	8 sampling points; Frequency: n.a.	[89]
Imidacloprid Acetamiprid	Surface water n.d. n.d. Serbia (n = 8)	Plastic bottles (1 L); Stored at 4 ºC. **<u>SPE</u>** **Cartridges**: Oasis® HLB (200 mg, 6 mL); **Conditioning:** 5 mL of methanol/dichloromethane (50/50, v/v) and 10 mL of deionized water; **Sample loading:** 250 mL; **Elution:** 10 mL of methanol/dichloromethane (50/50, v/v).	HPLC – MS/MS **Column type:** Zorbax Eclipse® XDB-C_{18} (75 mm x 4.6 mm i.d., 3.5 µm particle size); **Mobile phase:** water/methanol/acetic acid 10% (v/v) at gradient mode; **Flow rate:** 0.5 mL min^{-1}.	14 sampling points; Frequency: n.a.	[51]

Table 1. (Continued)

Watch List Compounds	Matrix Concentration Location (number of samples)	Sampling Procedure / Sample Preparation	Chromatographic Conditions	# Sampling Points Frequency of occurrence	Ref.
Thiacloprid Oxadiazon	Surface water n.a. n.a. Colombia (n = 13)	**SPE** **Cartridges**: Bond Elut C_{18} (500 mg, 6 mL); **Conditioning:** dichloromethane and methanol; **Sample loading:** 200 mL; **Elution:** 5 mL of ethyl acetate/dichloromethane (50/50, v/v).	UHPLC – QTOF/MS **Column type:** Acquity UHPLC BEH C_{18} (150 mm x 2.1 mm i.d., 1.7 µm particle size); **Mobile phase:** 0.01% formic acid/0.01% formic acid in methanol at gradient mode; **Flow rate:** 0.3 mL min^{-1}. GC–TOF/MS **Column type:** fused silica HP-5MS capillary column (30 m x 0.25 mm i.d., 0.25 µm); **Oven temperature:** 90 °C to 300 °C at 5 °C/min; **Volume injection:** 1 µL of sample; **Carrier gas:** Helium (1 mL min^{-1}).	13 sampling points; Thiacloprid: 23% Oxadiazon: 7%	[96]
Imidacloprid Thiacloprid Thiamethoxam Clothianidin Acetamiprid	Surface water 0.04 – 4.56 µg L^{-1} 0.02 – 1.37 µg L^{-1} 0.04 – 0.17 µg L^{-1} 0.02 – 0.42 µg L^{-1} 0.02 – 0.38 µg L^{-1} Australia (n = 39)	Stored in the dark at 4 ºC. **SPE** **Cartridges**: Sep-Pak Plus C_{18}; **Conditioning:** 5 mL of methanol + 5 mL of ultra-pure water; **Sample loading:** 250 mL; **Elution:** acetonitrile/methanol (2:1).	UHPLC – UV **Column type:** Zorbax SB-C_{18} (150 mm x 4.6 mm i.d., 5 µm particle size); **Mobile phase:** acetonitrile / water (25:75, v/v) **Flow rate:** 1 mL min^{-1}.	13 sampling points; Imidacloprid: 93% Thiacloprid: 80% (other compounds between 27% and 73%)	[66]

Table 1. (Continued)

Watch List Compounds	Matrix Concentration Location (number of samples)	Sampling Procedure / Sample Preparation	Chromatographic Conditions	# Sampling Points Frequency of occurrence	Ref.
Acetamiprid Clothianidin Imidacloprid Thiamethoxam	Surface water Up to 1.4 ng L^{-1} 1.0 – 12 ng L^{-1} Up to 25 ng L^{-1} 1.5 – 11 ng L^{-1} Japan (n = 52)	Glass bottles. **SPE** **Cartridges**: Oasis® HLB; **Conditioning:** methanol/ water (80/20, v/v); **Sample loading:** 500 mL; **Elution:** 5 mL of methanol.	HPLC – MS/MS **Column type:** TSK-GEL ODS-100V (150 mm x 2.0 mm i.d., 3 µm particle size); **Mobile phase:** water/methanol at gradient mode**;** **Flow rate:** 200 µL min^{-1}.	26 sampling points; Acetamiprid: 9.5 % Clothianidin: 100 % Imidacloprid: 95 % Thiamethoxam: 100 %	[97]
Imidacloprid	Surface water n.a. Argentina (n = 63)	Polypropylene bottles (1 L); Stored in the dark (-20 ºC). **SPE** **Cartridges**: Oasis® HLB (60 mg, 6 mL); **Conditioning:** 5 mL of methanol and 5 mL of nanopure water; **Sample loading:** 120 mL; **Elution:** 4 mL of methanol.	UHPLC – MS/MS **Column type:** Acquity UPLC BEH C_{18} column (100 mm x 2.1 mm i.d., 1.7 µm particle size); **Mobile phase:** ammonium acetate 3.6 mM and formic acid 5.22 mM aqueous solution/ acetonitrile modified with ammonium acetate 3.6 mM at gradient mode; **Flow rate:** 0.3 mL min^{-1}.	4 sampling points; Imidacloprid: 20 – 43%	[98]

Table 1. (Continued)

Watch List Compounds	Matrix Concentration Location (number of samples)	Sampling Procedure / Sample Preparation	Chromatographic Conditions	# Sampling Points Frequency of occurrence	Ref.
Imidacloprid Methiocarb	River water 2.34 – 6.14 ng L^{-1} Up to 391.44 ng L^{-1} Spain (n = 24)	Amber glass bottles (2 L), from the middle of the river width; Samples were transported in hermetic boxes refrigerated with ice and stored at 4 ºC. **SPE** **Cartridges**: Oasis® HLB (200 mg, 6 mL); **Conditioning:** 10 mL of dichloromethane/ methanol (50:50, v/v); **Sample loading:** 200 mL; **Elution:** 1 mL of methanol.	HPLC – MS/MS **Column type:** Luna C_{18} (150 mm x 2.1 mm i.d., 3 µm particle size); **Mobile phase:** 10 mM ammonium formate / methanol) (10 mM ammonium formate) at gradient mode; **Flow rate:** 0.4 mL min^{-1}.	24 sampling points; Imidacloprid: 58 % Methiocarb: n.a.	[99, 100]
Azithromycin Clarithromycin Erythromycin	Surface water n.d. Up to 5 ng L^{-1} 7 – 52 ng L^{-1} USA (n=4)	**SPE** **Cartridges**: Oasis® HLB (500 mg, 6 mL); **Conditioning:** 4 mL of methanol + 6 mL of HPLC-grade water; **Sample loading:** 200 mL; **Elution:** 5 mL of methanol.	HPLC –MS/MS **Column type:** Agilent Zorbax Eclipse Plus C_{18}, (100 mm x 2.1 mm i.d., 3.5 µm particle size); **Mobile phase**: 0.1% formic acid/acetonitrile at gradient mode; **Flow rate:** 0.2 – 0.3 mL min^{-1}.	8 sampling points; Azithromycin: n.a. Clarithromycin: n.a. Erythromycin: n.a.	[62]

Table 1. (Continued)

Watch List Compounds	Matrix Concentration Location (number of samples)	Sampling Procedure / Sample Preparation	Chromatographic Conditions	# Sampling Points Frequency of occurrence	Ref.
Diclofenac Azithromycin Erythromycin Clarithromycin	River water Up to 34.39 ng L^{-1} Up to 19.73 ng L^{-1} Up to 15.70 ng L^{-1} Up to 11.64 ng L^{-1} Spain (n = 13)	Amber glass bottles. **SPE** **Cartridges**: Oasis® HLB (60 mg, 3 mL); **Conditioning:** n.a.; **Sample loading:** 500 mL; **Elution:** 2 x 4 mL of methanol.	UHPLC – MS/MS **Column type:** $BEHC_{18}$, (100 mm x 2.1 mm i.d., 1.7 μm particle size); **Mobile phase**: 0.1% formic acid/acetonitrile, for diclofenac, at gradient mode; 10 mM ammonium acetate/ acetonitrile/methanol (50:50, v/v), for macrolide antibiotics, at gradient mode; **Flow rate:** 0.4 mL min^{-1}.	25 sampling points; Diclofenac: 77%, Azithromycin: 23%, Erythromycin: 92% Clarithromycin:100 %.	[67]
Diclofenac Erythromycin	River water 4.8 – 166 ng L^{-1} n.d. Romania (n = 20)	1 L bottle. **SPE** **Cartridges**: Strata®-X (200 mg, 6 mL); **Conditioning:** 6 mL methanol + 6 mL water; **Sample loading:** 200 mL; **Elution:** 6 mL of methanol.	UHPLC – MS/MS **Column type:** Acquity HSS T3, (100 mm x 2.1 mm i.d., 1.8 μm particle size); **Mobile phase**: water/methanol, both containing 2 mM ammonium and 160 μL L^{-1} formic acid at gradient mode; **Flow rate:** 0.4 mL min^{-1}.	20 sampling points; Diclofenac: n.a. Erythromycin: n.a.	[101]

Table 1. (Continued)

Watch List Compounds	Matrix Concentration Location (number of samples)	Sampling Procedure / Sample Preparation	Chromatographic Conditions	# Sampling Points Frequency of occurrence	Ref.
Diclofenac	River water 2.8 – 46 ng L^{-1} Spain (n = 267)	1-L polyethylene plastic vessels; Stored at 4 °C. **SPE** **Cartridges**: Strata®-X (60 mg, 3 mL); **Conditioning:** 4 mL of methanol and 4 mL of water; **Sample loading:** 500 mL; **Elution:** 8 mL of methanol.	HPLC – MS/MS **Column type:** Synergi Polar-RP 100A, (50 mm x 2.0 mm i.d., 2.5 μm particle size); **Mobile phase**: water/methanol, both with 0.1% formic acid, at gradient mode; **Flow rate:** 0.2 mL min^{-1}.	14 sampling points; Diclofenac: 26 %	[65]
Diclofenac	River water 18.4 – 156 ng L^{-1} Spain (n = 40)	1 L amber glass bottles. **SPE** **Cartridges**: Oasis® HLB (200 mg, 6 mL); **Conditioning:** 6 mL of methanol + 6 mL of Milli-Q water; **Sample loading:** 500 mL; **Elution:** 3 x 3 mL of methanol.	HPLC – MS/MS **Column type:** Zorbax SB-C_{18}, (42.1 mm x 30 mm i.d., 3.5 μm particle size); **Mobile phase**: 0.04% glacial acetic acid / acetonitrile at gradient mode; **Flow rate:** 0.5 mL min^{-1}.	10 sampling points; Diclofenac: 80 %	[102]

Table 1. (Continued)

Watch List Compounds	Matrix Concentration Location (number of samples)	Sampling Procedure / Sample Preparation	Chromatographic Conditions	# Sampling Points Frequency of occurrence	Ref.
Clarithromycin Diclofenac Erythromycin	River water 0.4 – 103 ng L^{-1} n.d. 1.1 – 808 ng L^{-1} China (n = 32)	Teflons FEP bottles (2L); Stored in a cooler. **SPE** **Cartridges**: Phenomenex Strata®-X (200 mg, 6 mL); **Conditioning:** 2 x 3 mL deionized water + 2 x 3 mL methanol; **Sample loading:** 250 mL; **Elution:** 2 x 3 mL of methanol.	HPLC – MS/MS **Column type:** Supelco Discovery HSC_{18} (150 mm x 4.6 mm i.d., 3 μm particle size); **Mobile phase:** 0.1% formic acid in water/acetonitrile at gradient mode; **Flow rate:** 0.3 mL min^{-1}.	16 sampling points; Clarithromycin: 94% Erythromycin: 94%	[103]
Clarithromycin Diclofenac	River water Up to 470 ng L^{-1} Up to 140 ng L^{-1} China (n = 119)	**SPE** **Cartridges**: Oasis® HLB Plus (225 mg, 6 mL); **Conditioning:** methanol + water acidified with hydrochloric acid (pH 4); **Sample loading:** 200 mL; **Elution:** 5 mL of methanol.	HPLC – MS/MS **Column type:** Atlantis T3 (100 mm x 2.1 mm i.d., 3 μm particle size); **Mobile phase:** water / acetonitrile, both with 0.1% formic acid, at gradient mode; **Flow rate:** n.a.	109 sampling points; Clarithromycin: 31% Diclofenac: 23%	[104]

Table 1. (Continued)

Watch List Compounds	Matrix Concentration Location (number of samples)	Sampling Procedure / Sample Preparation	Chromatographic Conditions	# Sampling Points Frequency of occurrence	Ref.
Erythromycin Diclofenac	River water Up to 60 ng L^{-1} n.d. South Africa (n = 16)	2.5 L amber bottles; Stored at 4 ºC. **SPE** **Cartridges**: Oasis® HLB (60 mg, 3 mL); **Conditioning:** 5 mL of methanol + 5 mL water adjusted to pH 4.20 with acetic acid; **Sample loading:** 300 mL; **Elution:** 10 mL of methanol + 5 mL of acetone.	HPLC – MS/MS **Column type:** Zorbax C_{18} (100 mm x 2.1 mm i.d., 3.5 µm particle size); **Mobile phase**: 0.1% acetic acid/acetonitrile at gradient mode; **Flow rate:** 0.25 mL min^{-1}.	8 sampling points; Erythromycin: n.a. Diclofenac: n.a.	[105]
Diclofenac Estradiol Estrone Ethinylestradiol	River water 165 – 886 ng L^{-1} 71 – 402 ng L^{-1} 59 – 1055 ng L^{-1} 116 – 333 ng L^{-1} Malaysia (n = 3)	White non-transparent plastic bottle (1 L); Transported at 4ºC. **SPE** **Cartridges**: Oasis® HLB (60 mg, 3 mL); **Conditioning:** 3 mL of MTBE (methyl-tert-butyl-ether) + 3 mL of methanol + 3 mL of acidified ultrapure water; **Sample loading:** 150 mL; **Elution:** 3 mL of a methanol/MTBE (10/90, v/v) + 3 mL of methanol.	HPLC – MS/MS **Column type:** Hypersil GOLD (50 mm x 2.1 mm i.d., 1.9 µm particle size); **Mobile phase**: 0.1% formic acid/methanol/acetonitrile at gradient mode; **Flow rate:** 0.1 mL min^{-1}.	4 sampling points; Diclofenac: n.a. Estradiol: n.a. Estrone: n.a. Ethinylestradiol: n.a.	[90]

Table 1. (Continued)

Watch List Compounds	Matrix Concentration Location (number of samples)	Sampling Procedure / Sample Preparation	Chromatographic Conditions	# Sampling Points Frequency of occurrence	Ref.
Diclofenac Azithromycin Clarithromycin Erythromycin	Surface water 15 – 292 ng L^{-1} Up to 18 ng L^{-1} 2 – 54 ng L^{-1} 0.8 – 47 ng L^{-1} Spain (n = 1)	**SPE** **Cartridges**: Oasis® HLB (200 mg, 6 mL); **Conditioning:** 5 mL of methanol + 5 mL of ultrapure water; **Sample loading:** 200 mL; **Elution:** 8 mL of methanol.	HPLC – MS/MS **Column type:** Acquity BEH C_{18} (100 mm x 2.1 mm i.d., 1.7 μm particle size); **Mobile phase**: 5 mM ammonium acetate: acetic acid (pH 5.2) / acetonitrile : methanol (2:1) at gradient mode; **Flow rate:** 0.4 mL min^{-1}.	3 sampling points; Diclofenac: n.a. Azithromycin: n.a. Clarithromycin: n.a. Erythromycin: n.a. .	[106]
Estradiol Estrone Ethinylestradiol	Surface water 2.2 – 39 ng L^{-1} Up to 25 ng L^{-1} n.d. Brazil (n = 5)	Amber glass bottles (1 L) **SPE** **Cartridges**: Oasis® HLB (500 mg, 6mL); **Conditioning:** 6 mL of methanol + 6 mL of water. **Sample loading:** 1000 mL; **Elution:** 2 x 3 mL of methanol.	HPLC – MS/MS **Column type:** Zorbax SB-C_{18} (30 mm x 2.1 mm i.d., 3.5 μm particle size); **Mobile phase**: water/methanol, both with 0.1% ammonium hydroxide, at gradient mode; **Flow rate:** 0.3 mL min^{-1}.	5 sampling points; Estradiol: n.a. Estrone: n.a. Ethinylestradiol: n.a.	[91]

Table 1. (Continued)

Watch List Compounds	Matrix Concentration Location (number of samples)	Sampling Procedure / Sample Preparation	Chromatographic Conditions	# Sampling Points Frequency of occurrence	Ref.
Diclofenac Estradiol Estrone Ethinylestradiol	River water 0.87 – 30 ng L^{-1} Up to 0.5 ng L^{-1} 0.20 – 4.2 ng L^{-1} Up to 1.0 ng L^{-1} South Korea (n = 6)	Amber glass bottles (2 L); Stored at 4 °C. **SPE** **Cartridges**: Oasis® HLB (200 mg, 5 mL); **Conditioning:** 5 mL of MTBE +5 mL of methanol + 5 mL of reagent water; **Sample loading:** 500 mL; **Elution:** 5 mL of methanol.	HPLC – MS/MS **Column type:** Luna C_{18} (150 mm x 4.6 mm i.d., 5 µm particle size); **Mobile phase**: 5 mM ammonium acetate/ methanol at gradient mode; **Flow rate:** 0.8 mL min^{-1}.	10 sampling points; Diclofenac: n.a. Estradiol: n.a. Estrone: n.a. Ethinylestradiol: n.a.	[92, 107]
Clarithromycin Erythromycin	Sea water Up to 0.08 ng L^{-1} Up to 1318 ng L^{-1} South China (n = 39)	Pre-cleaned bottles (5 L); Stored at 4 ºC. **SPE** **Cartridges**: Oasis® HLB (200 mg, 6mL); **Conditioning:** 10 mL methanol + 10 mL MilliQ water; **Sample loading:** 1000 mL; **Elution:** 12 mL of methanol.	HPLC – MS/MS **Column type:** Agilent Zorbax Eclipse Plus-C_{18} (100 mm x 2.1 mm i.d., 1.8 µm particle size); **Mobile phase**: 0.2% (v/v) formic acid aqueous solution with 2 mM ammonium acetate / /acetonitrile at gradient mode; **Flow rate:** 0.3 mL min^{-1}.	32 sampling points; Clarithromycin: n.a. Erythromycin: n.a.	[108]

Table 1. (Continued)

Watch List Compounds	Matrix Concentration Location (number of samples)	Sampling Procedure / Sample Preparation	Chromatographic Conditions	# Sampling Points Frequency of occurrence	Ref.
Diclofenac Azithromycin Erythromycin Imidacloprid Acetamiprid	River water n.a. 12 ng L^{-1} n.a. n.a. n.a. Serbia (n = 30)	Plastic bottle (1 L); Refrigerated without preservatives; Samples were taken from the middle course of the rivers, from a depth of about 1 m. **SPE** **Cartridges**: Oasis® HLB (200mg, 6 mL); **Conditioning:** 5 mL of methanol + 5 mL of deionized water + 5 mL of deionized water with pH adjusted to the value of water sample pH; **Elution:** 15 mL of methanol.	UHPLC – MS/MS **Column type:** Zorbax Eclipse® XDB–C_{18} (75 mm x 4.6 mm i.d., 3.5 μm particle size); **Mobile phase:** methanol / water / acetic acid (10%) at gradient mode; **Flow rate:** 0.6 mL min^{-1}.	5 sampling points were analysed; Diclofenac: n.a. Azithromycin: n.a. Erythromycin: n.a. Imidacloprid: n.a. Acetamiprid: n.a.	[4]
Diclofenac[1] Erythromycin[2]	River water Up to 3.6 ng L^{-1} Up to 11.4 ng L^{-1} Nigeria (n = 6)	Amber glass bottle (1 L). **SPE** **Cartridges**: Oasis® HLB (200 mg, 6 mL); **Conditioning:** 5 mL of 10 % (v/v) methanol/water; **Sample loading:** 400 mL; **Elution:** 5 mL of methanol.	HPLC – MS/MS **Column type:[1]** Thermo Scientific™ Hypersil GOLD™ C_{18} (100 mm x 2.1 mm, 3 μm); **Mobile phase**: a gradient of A - 5 mM ammonium acetate in acetonitrile : H_2O (10:90, v/v), pH 6; B – acetonitrile; **Column type:[2]** Thermo Scientific™ Betasil™ C_{18} (100 mm x 2.1 mm, 3 μm); **Mobile phase**: 5 mM ammonium formate / 0.1 % formic acid in methanol : H_2O at gradient mode; **Flow rate:** 0.45 mL min^{-1}.	10 sampling points; Diclofenac: 29 % Erythromycin: 14 %	[109]

Table 1. (Continued)

Watch List Compounds	Matrix Concentration Location (number of samples)	Sampling Procedure / Sample Preparation	Chromatographic Conditions	# Sampling Points Frequency of occurrence	Ref.
Azithromycin Erythromycin Clarithromycin	River water 0.3 - 1.0 ng L^{-1} 0.4 - 3.0 ng L^{-1} 0.2 - 13.1 ng L^{-1} Singapore (n = 8)	Amber glass bottle (2.5 L). **SPE** **Cartridges**: Supel™ Select HLB (60 mg, 3 mL); **Conditioning:** 10 mL of methanol + 6 mL of ultra-pure water; **Sample loading:** 500 mL; **Elution:** 6 mL of methanol.	HPLC-MS/MS **Column type:** Agilent Poroshell 120 SB-C_{18} (50 mm x 2.1 mm i.d., 2.7 μm particle size); **Mobile phase**: 5 mM ammonium acetate buffer / acetonitrile : methanol (1:1) at gradient mode; **Flow rate:** 0.5 mL min^{-1}.	4 sampling points; Azithromycin: n.a. Erythromycin: n.a. Clarithromycin: n.a.	[110]
Erythromycin	Surface water Up to 372 ng L^{-1} China (n = 360)	Polypropylene bottles (1 L); Stored at 4 ºC. **SPE** **Cartridges**: Oasis® HLB (200 mg, 6 mL); **Conditioning:** 5 mL of methanol + 5 mL of pure water; **Sample loading:** 200 mL; **Elution:** 6 mL of methanol with 5% ammonium hydroxide.	HPLC-MS/MS **Column type:** XTerra MS C_{18} (100 mm x 2.1 mm i.d., 3.5 μm particle size); **Mobile phase**: methanol: acetonitrile (50:50, v/v) / 0.3% formic acid in water (containing 0.1% ammonium formate) at gradient mode; **Flow rate:** 0.2 mL min^{-1}.	36 sampling points; Erythromycin: 98 %	[111]

Abbreviations: DAD, diode array detection; EDTA, Ethylenediaminetetraacetic acid; GC–TOF/MS, gas chromatography time of flight mass spectrometry; HPLC, high performance liquid chromatography; MS/MS, tandem mass spectrometry; MTBE, methyl-tert-butyl-ether; n.a., not available; n.d., not detected; QTOF/MS, quadrupole-time of flight mass spectrometry; UHPLC, ultra high performance liquid chromatography; UV, ultraviolet.

3. Analytical method development for determination of CECs in surface water

3.1. Chemicals and materials

Table 2 shows the group, substance, structure, CAS number (Chemical Abstracts Service registry number), molecular weight (Mw), solubility and pKa of Watch List compounds that were studied in this dissertation. All reference standards (17-beta-estradiol, estrone, 17-alpha-ethinylestradiol, diclofenac sodium, 2,6-di-tert-butyl-4-methylphenol, 2-ethylhexyl 4-methoxycinnamate, erythromycin, clarithromycin, azithromycin dehydrate, methiocarb, imidacloprid solution 100 ng μL^{-1} in acetonitrile, thiacloprid, thiamethoxam, clothianidin, acetamiprid, oxadiazon and triallat) were purchased from Sigma–Aldrich (Steinhein, Germany). Except imidacloprid, each standard was dissolved in methanol to achieve stock solutions with a concentration of approximately 1000 mg L^{-1}. A working standard solution containing 300 ng L^{-1} of 17-beta-estradiol, estrone, 17-alpha-ethinylestradiol, diclofenac, 2,6-di-tert-butyl-4-methylphenol, 2-ethylhexyl 4-methoxycinnamate, erythromycin, clarithromycin, azithromycin, methiocarb, imidacloprid, thiacloprid, thiamethoxam, clothianidin, acetamiprid, oxadiazon and triallat was prepared by dilution in methanol, to optimize the SPE and UHPLC-MS/MS analysis. A working standard solution containing 200 ng L^{-1} of diclofenac, 2-ethylhexyl 4-methoxycinnamate, clarithromycin, azithromycin, methiocarb, imidacloprid, thiamethoxam and acetamiprid and 800 ng L^{-1} of erythromycin, thiacloprid and clothianidin was prepared by dilution in methanol to validate the method. Deuterated compounds used as internal standards (diclofenac-d4, azithromycin-d3, methiocarb-(N-methyl-d3), clothianidin-d3 and acetamiprid-d3) were also purchased from Sigma–Aldrich (Steinhein, Germany). A solution containing 10 mg L^{-1} of each internal standard was prepared by dilution in methanol.

Methanol and acetonitrile (MS grade) were purchased from VWR International (Fontenay-sous-Bois, France). Ethanol (HPLC grade) was acquired from Fisher Scientific UK Limited (Leicestershire, United Kingdom). Ammonium acetate, formic acid, ammonium hydroxide 25% and sulphuric acid were acquired from Merck (Darmstadt, Germany). Ultrapure water was supplied by a Milli-Q water system. HPLC grade solvents were filtered with 0.22 µm nylon membrane filters (Membrane Solutions, Texas, USA). For SPE, the cartridges tested were Oasis® HLB (Hydrophilic-Lipophilic-Balanced), Oasis® MAX (Mixed-mode Anion-eXchange) and Oasis® MCX (Mixed-mode Cation eXchange) (150 mg, 6 mL) purchased from Waters (Milford, MA, USA). The pH measurements were performed with a pH meter pHenomenal® pH 1100 L (VWR, Germany).

Table 2. Watch List compounds: group, CAS Number, molecular weight (Mw), structure, solubility and pKa.

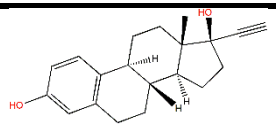
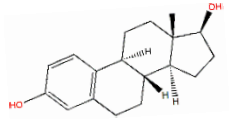
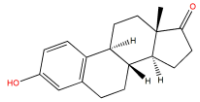
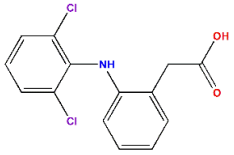
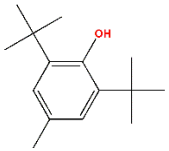
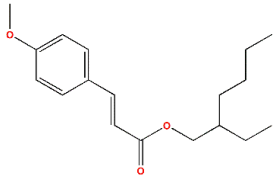
Group	Substance	CAS Number	Mw (g mol^{-1})	Structure	Solubility (mg L^{-1})	pKa
Hormones	17-Alpha-ethinylestradiol (EE2)	57-63-6	296.40		11.3 (27 °C)	10.33
	17-Beta-estradiol (E2)	50-28-2	272.38		3.6 (27 °C)	10.71
	Estrone (E1)	53-16-7	270.37		30 (25 °C)	10.77
Anti-inflammatory	Diclofenac	15307-86-5	296.15		2.4 (25 °C)	4.15
Food additive	2,6-Ditert-butyl-4-methylphenol	128-37-0	220.35		0.4 (20 °C)	12.23
Organic UV filter	2-Ethylhexyl 4-methoxycinnamate	5466-77-3	290.40		0.2 (20 °C)	2.9

Table 2. (Continued)

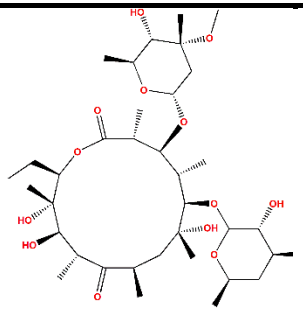
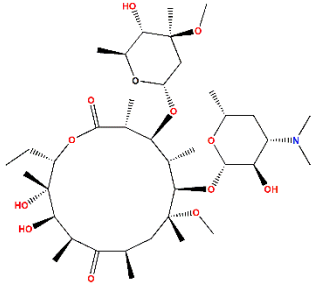
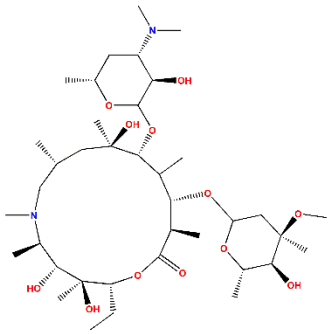
Group	Substance	CAS Number	Mw (g mol^{-1})	Structure	Solubility (mg L^{-1})	pKa
Macrolide antibiotics	Erythromycin	114-07-8	733.93		2000 (28 °C)	8.90
	Clarithromycin	81103-11-9	747.95		1.7 (25 °C)	8.99
	Azithromycin	83905-01-5	748.98		2.4 (25 °C)	8.74

Table 2. (Continued)

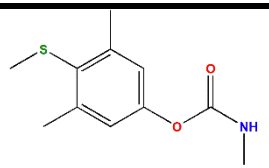
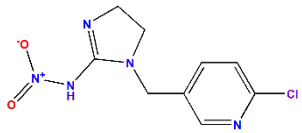
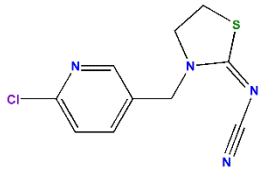
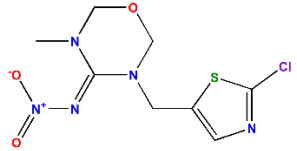
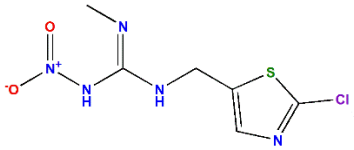
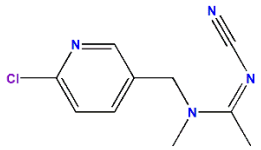
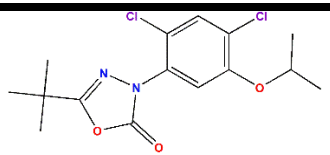
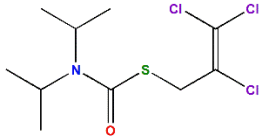
Group	Substance	CAS Number	Mw (g mol^{-1})	Structure	Solubility (mg L^{-1})	pKa
Pesticide	Methiocarb	2032-65-7	225.31		27 (20 °C)	12.2
Neonicotinoids	Imidacloprid	105827-78-9	255.66		610 (20 °C)	1.56
	Thiacloprid	111988-49-9	252.72		185 (20 °C)	1.62
	Thiamethoxam	153719-23-4	291.71		4100 (25 °C)	0.41
	Clothianidin	210880-92-5	249.68		327 (20 °C)	11.09
	Acetamiprid	135410-20-7	222.67		4250 (25 °C)	0.70

Table 2. (Continued)

Group	Substance	CAS Number	Mw (g mol^{-1})	Structure	Solubility (mg L^{-1})	pKa
Herbicide	Oxadiazon	19666-30-9	345.22		0.7 (24 °C)	n.a.
	Triallat	2303-17-5	304.66		2.0 (25 °C)	n.a.

Abbreviations: CAS number, Chemical Abstracts Service Registry *Number;* Mw, molecular weight; n.a., not available; pKa, acid dissociation constant.

3.2. Solid-phase extraction

Surface water from the source of Sousa River (41.371785, -8.167365) was collected and used as blank matrix for the SPE optimization and method development and validation. Figure 6 shows a general procedure of SPE.

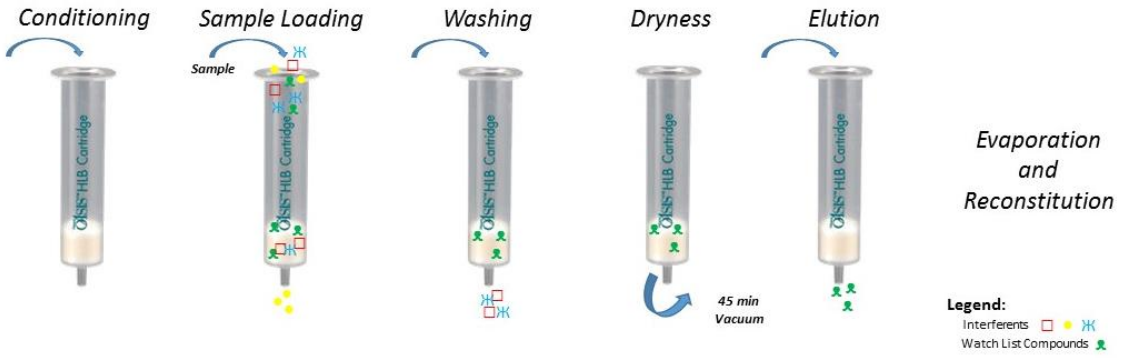


Figure 6. Schematic representation of SPE procedure.

SPE was performed using a LiChrolut® vacuum manifold, purchased from VWR (Merck Millipore, Billerica, MA, USA) and Oasis® HLB, MCX and MAX cartridges (150 mg, 6 mL) were tested. Solvents, sample volume and pH were also optimized. The conditioning step was based on the addition of 4 mL of an organic solvent (methanol for MAX and MCX; methanol, ethanol or acetonitrile for HLB) followed by 4 mL of ultrapure water, both at a flow rate of 1 mL min^{-1}. 250 mL of surface water blanks and spiked samples (300 ng L^{-1}) were loaded through the cartridges at a constant flow rate using the extraction device (Figure 7) connected to a vacuum pump from VWR VP 100 (Merck Millipore, Billerica, MA, USA).

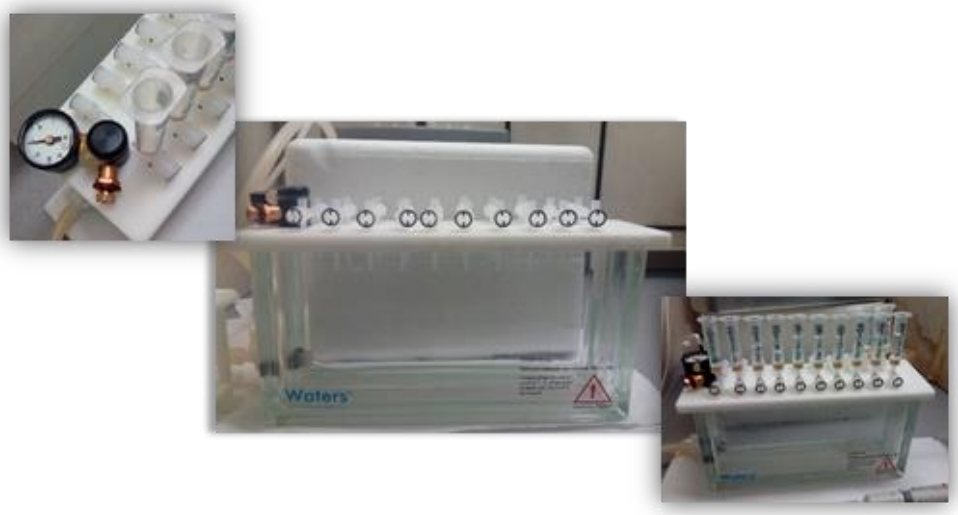


Figure 7. Manifold used to SPE procedure.

Before loading, samples were filtered through 1.2 µm glass microfiber filters GF/C, 47 mm (Whatman™, United Kingdom) and the pH was adjusted to 3 using sulphuric acid for MCX cartridges or adjusted to 11 using ammonium hydroxide, in the case of MAX cartridges. For SPE experiments using HLB cartridges, pH 3, 7 and 11 were tested. Then, a washing step was performed with 4 mL of ultrapure water, 5% ammonium hydroxide aqueous solution or 2% formic acid aqueous solution, for HLB, MAX and MCX, respectively. Before elution, cartridges were dried under vacuum aspiration for 45 min. MAX and MCX elutions were performed in two-steps, the first elution using 4 mL of methanol to extract the neutral compounds and weak acid compounds (MCX) or weak basic compounds (MAX), and the second one using a methanolic solution of 5% ammonium hydroxide to elute the basic compounds (MCX) or a methanolic solution of 2% formic acid to elute the acidic compounds (MAX). HLB cartridges were eluted in a single step performed with 4 mL methanol, acetonitrile or ethanol. The extracts were evaporated to dryness with a CentriVap® Concentrator, LABCONCO® unit.

For reconstitution, the residues were dissolved in 250 µL of ethanol, methanol or acetonitrile, depending on the solvent used for SPE. The extracts were then filtered by using 0.22 µm polytetrafluoroethylene (PTFE) syringe filters (Membrane Solutions, Texas, USA). Breakthrough volume was evaluated using HLB cartridges and ethanol as solvent, testing four different sample volumes (100, 250, 500 and 1000 mL) adjusted to pH 3, using both blank samples and spiked samples to determine the adequate volume to achieve the highest recovery.

3.3. Ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) parameters

Chromatographic analysis was performed using a Shimadzu Corporation apparatus (Tokyo, Japan) (Figure 8). It consists on an UHPLC equipment (Nexera) with two pumps (LC-30AD), an autosampler (SIL-30AC), an oven (CTO-20AC), a degasser (DGU-20A 5R) and a system controller (CBM-20A) coupled to a triple quadrupole mass spectrometer detector (Ultra Fast Mass Spectrometry series LCMS-8040) with the software LC Solution Version 5.41 SP1. A Kinetex™ 1.7 µm XB-C18 100 Å column (100 × 2.1 mm, i.d.), supplied by Phenomenex Inc. (California, USA) was used.

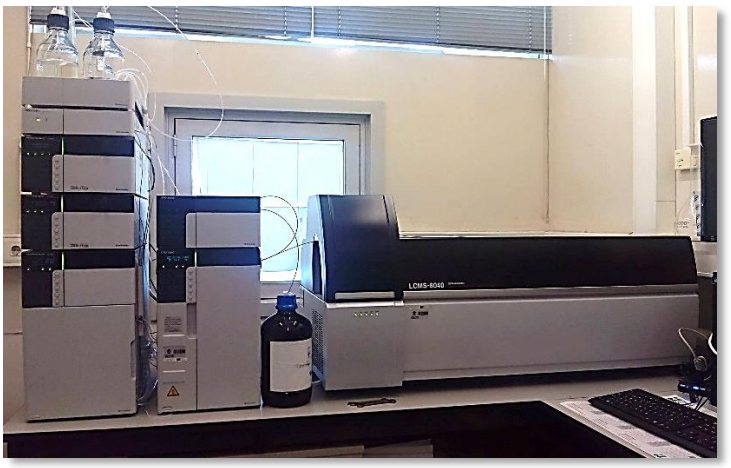


Figure 8. Equipment used for LC–MS/MS analysis.

The optimized mobile phase was methanol/water (75/25, v/v) performed at gradient mode at a flow rate of 0.25 mL min^{-1}. The mobile phase was programmed as follows: 75% for 0.80 min; a linear gradient from 75 % to 100 % in 0.8 min (held for 4.90 min), a linear gradient from 100 % to 75 % in 0.10 min and finally an equilibration time of 3 min, with a total run time of 10 min. Column oven and autosampler temperatures were set at 35 and 4 ºC, respectively. An electrospray ionization source was used, operating in both positive and negative ionization modes. Volume of injection was 10 µL. Each individual standard solution at 1000 µg L^{-1} was directly injected into the MS detector in order to select the precursor ion through full scan mode, to choose the most abundant fragments and to optimize the mass spectrometer parameters for each compound, namely: i) the declustering potential, ii) collision energy and iii) collision cell exit potential. Two selected reaction monitoring (SRM) transitions between the precursor ion and the two most abundant fragment ions of each compound were selected. For chromatographic analysis, the most abundant fragment ion was used as quantifier and the second most abundant as qualifier, with a scan time of 100 ms/transition. Parameters, such as capillary voltage (1.5, 2.5, 3.5 and 4.5 kV), drying gas flow (10.0, 12.5, 15.0 dm^3 min^{-1}), nebulizing gas flow (1.0, 1.5, 2.0, 2.5 and 3.0 dm^3 min^{-1}), desolvation temperature (200, 225, 250 and 300 ºC) and source temperature (250, 300, 350, 400 and 450 ºC) were optimized. Argon was used as collision induced dissociation (CID) gas at 230 kPa.

3.4. Method parameters and validation

The method validation was performed according to previous works [72] and international guidelines [112], considering the following parameters: selectivity, recovery, linearity and range, limits of detection and quantification, precision and accuracy. Selectivity was verified by comparing the chromatograms of standards extracted from the spiked and non-spiked (blank) surface water samples and standards dissolved in methanol. Three quality control (QC) standard

solutions were used for recovery assays and prepared in triplicate for three consecutive days. Such QC solutions consisted in surface water spiked at three different concentrations: 4.5, 45 and 90 ng L^{-1} for diclofenac, 2-ethylhexyl 4-methoxycinnamate, clarithromycin, azithromycin, methiocarb, imidacloprid, thiamethoxam and acetamiprid; 18, 180 and 360 ng L^{-1} for erythromycin, thiacloprid and clothianidin. Recovery of the method was obtained by comparing peak areas of the standards extracted from the spiked matrix (subtracting the blank signal) with peak areas of similar concentrations of methanolic standard solutions. Linearity and range were evaluated using the internal calibration. Triplicates of 500 mL surface water samples adjusted at pH 3 were spiked with eight different standard concentrations: 1.0, 2.5, 5.0, 10, 25, 50, 75 and 100 ng L^{-1} for all compounds, except for erythromycin, thiacloprid and clothianidin, which concentrations were 4.0, 10, 20, 40, 100, 200, 300 and 400 ng L^{-1}. Before extraction by SPE, 20 µL of internal standards solution at 10 mg L^{-1} was added to each sample. The optimized SPE procedure was performed and the extracts were reconstituted in 250 µL of ethanol to perform the calibration curves, by injecting 10 µL in the UHPLC equipment. Method detection (MDL) and quantification (MQL) limits were calculated for each studied compounds from equations 1 and 2, respectively [112].

$$MDL = \frac{3.3 \times \sigma}{S} \qquad (1)$$

$$MQL = \frac{10 \times \sigma}{S} \qquad (2)$$

where:

σ is the standard deviation of the response;

S is the slope of the calibration curve.

The slope S was estimated from the calibration curve of each analyte. The estimate of σ was carried out based on the standard deviation of the blank. Measurement of the standard deviation of the blank was performed by determining the area ratio between each analyte and the related internal standard in six replicates of blank samples [112]. Instrument detection limit (IDL) and instrument quantification limit (IQL) were performed through multiplication of the respective method limit by the pre-concentration factor. The three QC standard solutions were also analysed to evaluate the accuracy and intra and inter-batch precision. Precision of the method was expressed through the relative standard deviation (RSD) of the replicate measurements [72]. Accuracy was determined as the percentage of agreement between the concentrations of the standards analysed in the SPE extracts and the nominal concentration. For the purpose of assessing possible carry-out effect, ethanol was injected after each triplicates.

3.5. Matrix effect evaluation

The matrix effect was assessed by the post-extraction addition method [72]. The signal of post-spiked extracts of blank surface water samples (45 µg L^{-1} for all compounds, except for erythromycin, thiacloprid and clothianidin, which concentration was 180 µg L^{-1}) after subtracting the signal of extracts of non-spiked surface water samples, was analysed and compared with the signal of standard ethanolic solutions. The matrix effect (ME) ratio was obtained by the equation [72]: ME (%) = A/B × 100, where A is the average area obtained for extracts of blanks spiked after extraction subtracted from average area of the non-spiked blanks and B is the average area of the compounds in an ethanolic solution and at the same concentration as the post-spiked extracts. A value lower than 100% indicates an ionization suppression; equal to 100% indicates the absence of matrix effect; higher than 100% indicates an ionization enhancement.

3.6. Water sampling

Sampling strategy was designed based mainly on the presence of tributaries and DWTPs/WWTPs that probably could affect the target Rivers. The sample collection was made at the spring season of 2016 (May and June) using a bottle sampler, at different locations of the middle of the river in order to get representative samples. Afterwards, water samples were transferred to a 1 L amber glass bottle, refrigerated at 4 ºC and processed within 24 h for CECs, turbidity, dissolved organic carbon (DOC) and ions determination. Certain physical-chemical parameters were analysed on site, in each sampling point, such as: pH, conductivity, salinity, oxidation-reduction potential, temperature, dissolved oxygen and total dissolved solids. These parameters were measured using a HI98194 Multiparameter Meters, HANNA® instruments. Flow rate was estimated by measuring the velocity of water and the sampling points sections (depth at three sites and length). Turbidity, DOC and ions concentrations, were measured at the lab. Surface water samples were used as collected to measure turbidity, using a HI88703 Turbidimeter, HANNA® instruments. For DOC, ions and CECs determination, samples were previously filtered by 1.2 µm glass microfiber filters GF/C, 47 mm (Whatman™, United Kingdom). DOC was analysed in a TOC-L Shimadzu® instrument. Ion chromatography analyses were performed in a Metrohm 881 Compact IC Pro apparatus, equipped with a Metrosep C4 Cationic Exchange Column (250 mm x 4.0 mm) for quantification of sodium, ammonium, potassium, calcium and magnesium operating at 25 ºC and a Metrosep A Supp 7 Anionic Exchange Column (250 mm x 4.0 mm) for quantification of bromates, chlorides, bromides, nitrites, nitrates and sulfates, operating at 45 ºC. The selected eluents were 3.6 mmol L^{-1} Na_2CO_3, 100 mmol L^{-1} H_2SO_4 and ultra-pure water for cations analysis and 1.7 mmol L^{-1} nitric acid and 0.7 mmol L^{-1} dipicolinic acid for anions analysis. For each analysis, 20 µL of sample was injected. The filtered sample used to assess the CECs concentration was acidified before SPE procedure. Figure 9 shows a summary of the sampling procedure.

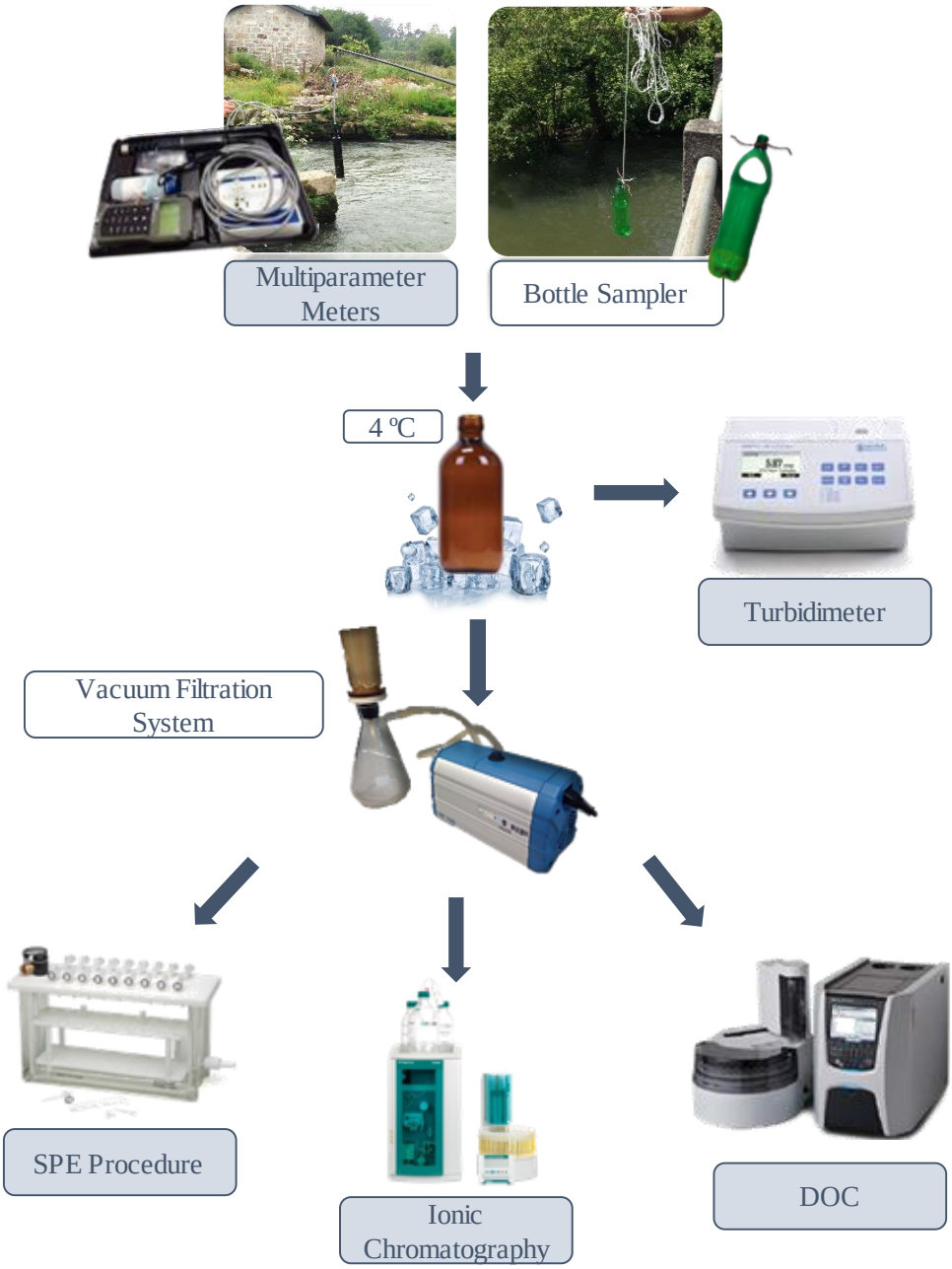


Figure 9. Summary of sampling procedure.

3.7. Monitoring sites

The sampling campaigns were performed in the Sousa River at 18th May and in the Ave River at 1st June, 2016. Sousa River was studied along the whole course, whereas Ave River was mainly studied after the Ermal Dam. Sampling point P1 is the average of 5 points before the Ermal dam.

3.7.1. Sousa River

Figure 10 illustrate the sampling points chosen for Sousa River and the respective GPS coordinates.

Target Point	Lat. (ºN)	Long (ºW)
Source	41.371785	-8.167365
P1	41.306724	-8.219165
P2	41.299408	-8.217599
P3	41.290617	-8.235088
P4	41.238516	-8.277059
P5	41.223998	-8.290396
P6	41.195955	-8.328980
P7	41.189453	-8.336823
P8	41.170067	-8.348392
P9	41.159823	-8.369752
P10	41.133197	-8.418973
P11	41.128939	-8.433870
P12	41.105393	-8.472120
P13	41.093909	-8.501425
P14	41.087793	-8.519653

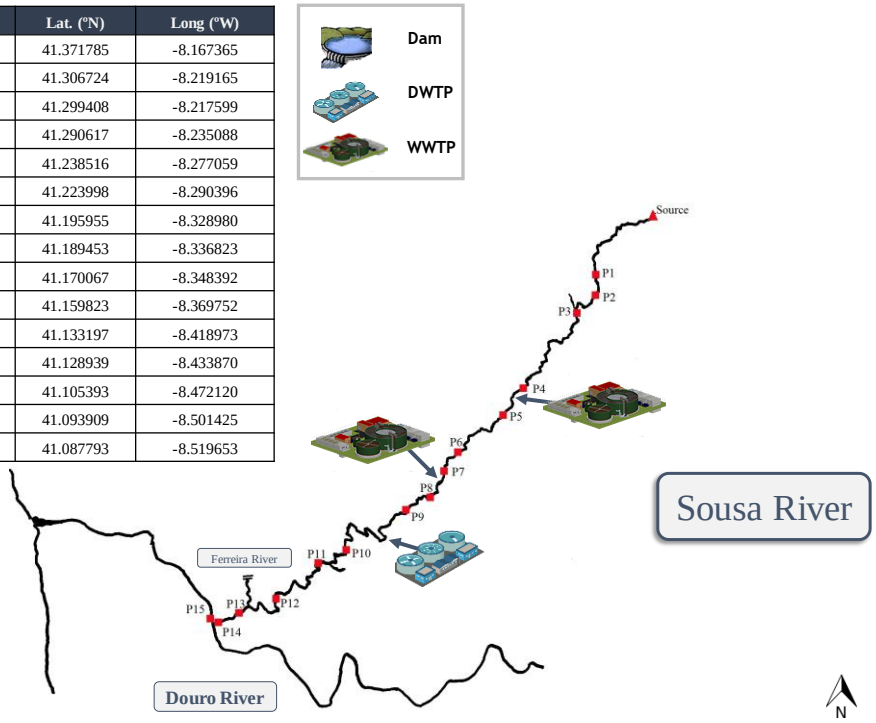


Figure 10. Sampling points of Sousa River and respective GPS coordinates.

3.7.2. Ave River

Figure 11 illustrate the sampling points chosen for Ave River and the corresponding GPS coordinates.

Target Point	Lat. (ºN)	Long (ºW)
P1.1	41.578277	-8.130229
P1.2	41.579992	-8.113193
P1.3	41.596798	-8.123149
P1.4	41.605776	-8.135009
P1.5	41.595495	-8.147004
P2	41.576986	-8.166351
P3	41.560289	-8.214434
P4	41.539299	-8.263313
P5	41.505453	-8.306600
P6	41.471767	-8.346705
P7	41.420384	-8.377359
P8	41.389509	-8.396709
P9	41.346214	-8.470378
P10	41.351377	-8.681185
P11	41.351100	-8.739360

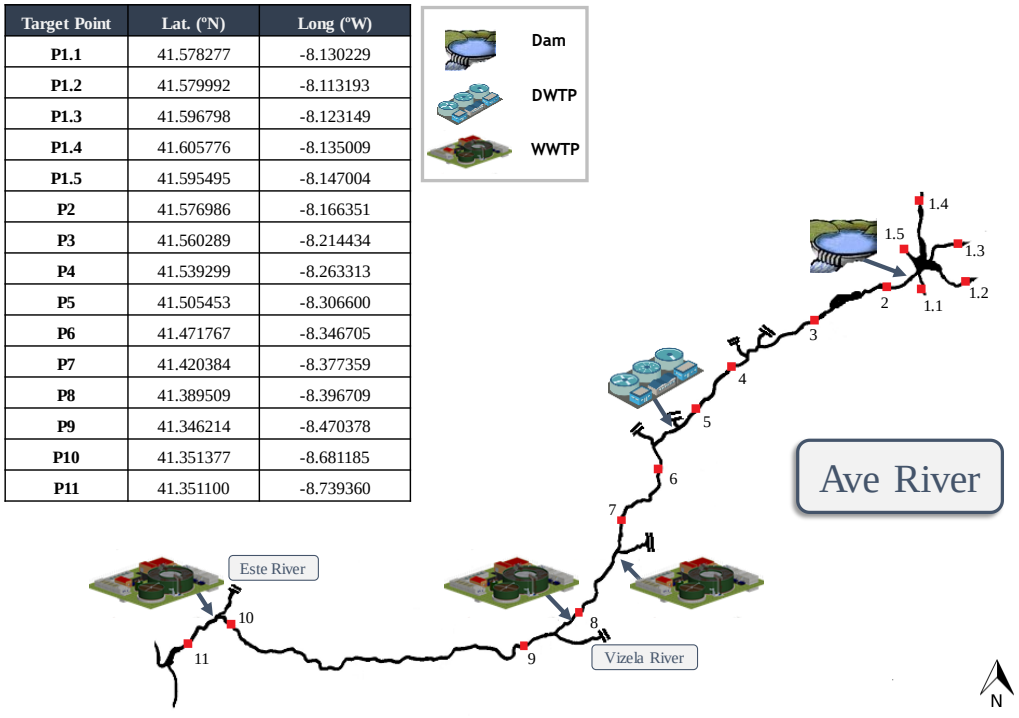


Figure 11. Sampling points of Ave River and respective GPS coordinates.

4. Results and discussion

4.1. UHPLC-MS/MS

4.1.1. Chromatographic separation

Decision 2015/495/EU recommended different chromatographic methods for the determination of Watch List compounds in surface water [29]. Despite the attempts performed, 2,6-ditert-butyl-4-methylphenol, oxadiazon and triallat were not included in developed UHPLC-MS/MS method due to their physical-chemical characteristics that make them more prone to be analyzed by GC-MS, which is the "indicative analytical method" of Decision 495/2015/EU. The chromatographic separation was performed using a Kinetex™ 1.7 µm XB-C18 Å column (100 x 2.1 mm, i.d.). In order to ensure the inclusion of all the compounds whether possible, the focus of the chromatographic optimization process was privileging the better results found for compounds with lower signal intensity. This type of column has a nonpolar stationary phase and has affinity and selectivity to polar and moderately nonpolar compounds, working as reversed-phase when using a more polar mobile phase. Thereby, nine combinations of mobile phases were tested, being methanol, ethanol and acetonitrile chosen as organic phases and ultrapure water, 10 mM of ammonium acetate or 0.1% of formic acid tested as aqueous phases (Figure 12), initially at isocratic mode with a flow rate of 0.20 mL min^{-1} and 70/30 (v/v), respectively.

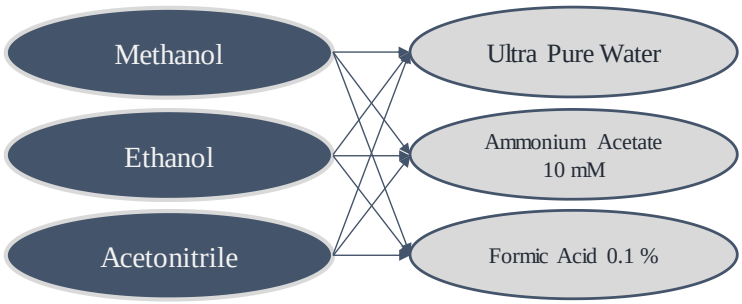


Figure 12. Combination of organic and aqueous phases tested.

Regarding the tested organic phases, acetonitrile led to higher signal for 17-beta-estradiol, estrone and 17-alpha-ethinylestradiol; however, it would compromise the detection of most Watch List compounds, which response was weak. The contrary was observed for ethanol and methanol, both leading to a low intensity for determination of the estrogens. Thus, the method development included the other 11 CECs. Comparing ethanol and methanol, the latter was the organic phase which gave the best signal for the largest number of compounds. Both ammonium acetate and formic acid did not improve the peak shape and resolution, comparing with ultrapure water. Therefore, the selected mobile phase was a mixture of methanol and ultrapure water (75/25, v/v) at gradient mode. Other parameters were also optimized, such as the flow rate that was set at 0.25 mL min^{-1}, the temperature of the column oven (35 ºC) and injection volume that was set at 10 µL.

Total run time including column reequilibration was 10 min. Samples were refrigerated in the autosampler at 10 ºC. Table 3 shows the gradient mode applied. Appendix B – Figure B shows an example of a chromatogram of the target analytes obtained with the optimized mobile phase.

Table 3. Optimized gradient mode.

Time	Methanol	Water
0.0	75 %	25 %
0.8	75 %	25 %
1.6	100 %	0 %
6.5	100 %	0 %
7.0	75 %	25 %
10.0	75 %	25 %

4.1.2. Mass spectrometry (MS / MS)

A method of electrospray ionization tandem MS was developed for quantitative analysis. The triple quadrupole MS/MS detection has the ability to simultaneously quantify target analytes at trace levels and provide their identity confirmation. Thus, precursor ion for each Watch List compound was selected through the single direct injection in full scan mode. All Watch List compounds were scanned in both ESI (+) and ESI (−) ionization modes. Most of the target analytes were detected in the positive ionization mode with the exception of diclofenac which was detected using the negative ionization mode. For all the studied compounds (diclofenac, EHMC, erythromycin, clarithromycin, azithromycin, methiocarb, imidacloprid, thiacloprid, thiamethoxam, clothianidin and acetamiprid) two selected reaction monitoring (SRM) transitions between the precursor ion and the two most abundant fragment ions were selected and monitored. The most abundant product ion from each precursor ion (SRM1) was selected for quantification, and the second most abundant transition (SRM2) was used for identity confirmation. This allowed the accomplishment of the requirements established by EU Commission Decision 2002/657/EC [113] related to identification and confirmation of micropollutants analysed by LC–MS/MS. The ionization mode, two SRMs, declustering potential (DP), collision energy (CE), collision cell exit potential (CXP) and ion ratio for each compound are shown in Table 4. Retention times were also used for confirmation of the identity of the compounds and are shown in Table 5.

Table 4. Selected reaction monitoring (SRM) instrument parameters for tandem mass spectrometry analysis of target analytes.

Group	Analyte	IS set[a]	ESI mode (NI[b] or PI[c])	Precursor ion (m/z)	Quantification (SRM1)				Confirmation (SRM2)				Ion ratio (± SD)
					Product Ion	DP[d] (V)	CE[e] (V)	CXP[f] (V)	Product Ion	DP[d] (V)	CE[e] (V)	CXP[f] (V)	
Anti-inflammatory	Diclofenac	1	NI	294.1	249.95	20	12	26	213.95	20	21	21	26.27 (± 0.17)
	Diclofenac-d4 (1)		NI	297.9	254.05	14	12	28	-	-	-	-	n.a.
Organic UV Filter	2-Ethylhexyl 4-methoxycinnamate	2	PI	291.2	179.1	-14	-9	-18	161.1	-14	-19	-15	1.01 (± 0.07)
Macrolide Antibiotics	Erythromycin	2	PI	734.4	158.15	-36	-34	-30	576.35	-36	-21	-28	2.28 (± 0.18)
	Clarithromycin	2	PI	748.4	158.15	-40	-30	-15	83.2	-40	-53	-30	3.06 (± 0.19)
	Azithromycin	2	PI	749.4	158.15	-38	-44	-28	83.15	-38	-54	-29	1.65 (± 0.13)
	Azithromycin-d3 (2)		PI	752.4	158.05	-38	-47	-14	-	-	-	-	n.a.
Pesticide	Methiocarb	3	PI	226.1	169.1	-24	-9	-17	121.1	-24	-19	-21	1.13 (± 0.16)
	Methiocarb-d3 (3)		PI	229.1	169.1	-25	-11	-30	-	-	-	-	n.a.
Neonicotinoids	Imidacloprid	4	PI	255.7	209.05	-30	-15	-21	175.05	-30	-18	-17	0.58 (± 0.31)
	Thiacloprid	4	PI	252.9	126	-28	-21	-21	99	-28	-44	-17	5.50 (± 0.17)
	Thiamethoxam	4	PI	291.9	211.1	-30	-14	-21	181.05	-30	-24	-17	2.98 (± 0.18)
	Clothianidin	5	PI	249.9	132	-29	-15	-23	169.05	-29	-13	-16	1.34 (± 0.15)
	Clothianidin-d3 (5)		PI	252.9	172	-29	-13	-17	-	-	-	-	n.a.
	Acetamiprid	4	PI	222.7	126	-15	-20	-23	56.1	-15	-16	-22	2.60 (± 0.19)
	Acetamiprid-d3 (4)		PI	226.1	126	-24	-21	-23	-	-	-	-	n.a.

[a] IS, internal standard; [b] NI, negative ionization mode; [c] PI, positive ionization mode; [d] DP, declustering potential; [e] CE, collision energy; [f] CXP, collision cell exit potential;
[g] n.a, not applicable.

Desolvation and source temperatures, nebulizing and drying gas flows and capillary voltage were also optimized by injecting a standard solution with target analytes and comparing the signal obtained. The results of these parameters optimization are shown in Appendix C in Figures C1, C2, C3, C4 and C5. Thus, the best conditions for MS parameters were: 3.0 dm^3 min^{-1} for nebulizing gas flow, 12.5 dm^3 min^{-1} for drying gas flow, 4.5 kV for capillary voltage, 250 ºC for desolvation temperature and 400 ºC for source temperature.

4.2. Solid-phase extraction optimization

4.2.1. Cartridges

Oasis® HLB cartridges contain a versatile sorbent which is suitable for a wide spectrum of compounds due to its hydrophilic/lipophilic retention modes. However, other two types of cartridges were assessed, namely MAX (mixed-mode, reversed-phase/strong anion-exchange) and MCX (mixed-mode, reversed-phase/strong cation-exchange), mainly used for extraction of acidic compounds and basic compounds, respectively. The experiments were performed using methanol as conditioning and eluting solvent for Oasis® HLB cartridges and sample pH adjusted to 3, according to the literature [67, 101]. The results obtained were compared and are shown in Figure 13.

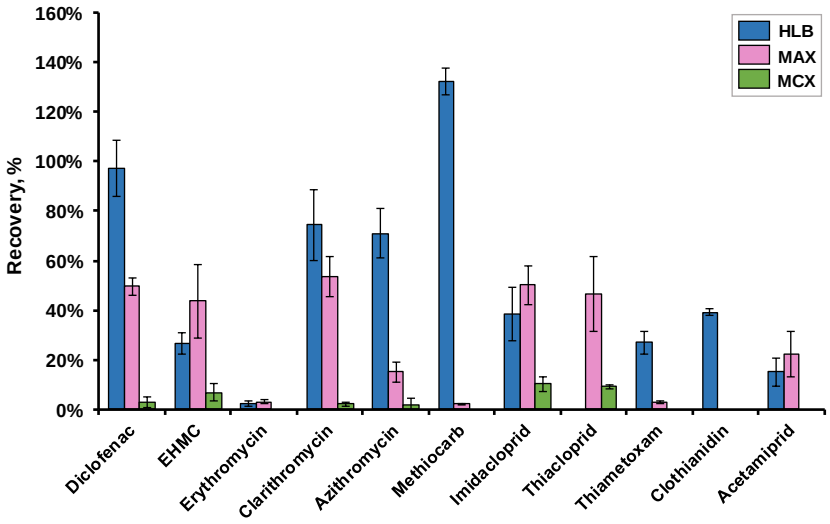


Figure 13. Recoveries obtained for Watch List compounds for different cartridges (Oasis® HLB, MAX and MCX) extracting 250 mL of surface water samples (pH 3 for HLB and MAX; pH 11 for MCX) and using methanol as solvent.

In general, higher recoveries were obtained for Oasis® HLB that were chosen for the next experiments, although for a small group of compounds including EHMC, imidacloprid, thiacloprid and acetamiprid, the Oasis® MAX gave superior recoveries. These results were expected, due to the low pKa of such compounds.

4.2.2. Sample pH

The evaluation of the optimal pH value of the water samples in SPE optimization is crucial, since it can influence substantially the extraction efficiency. Thus, preliminary tests adjusting samples to different pH (3, 7 and 11) were performed to choose the value that provided good recoveries by extracting 250 mL of surface water samples through Oasis® HLB, using methanol as conditioning and eluting solvent. The achieved recoveries are shown in Figure 14.

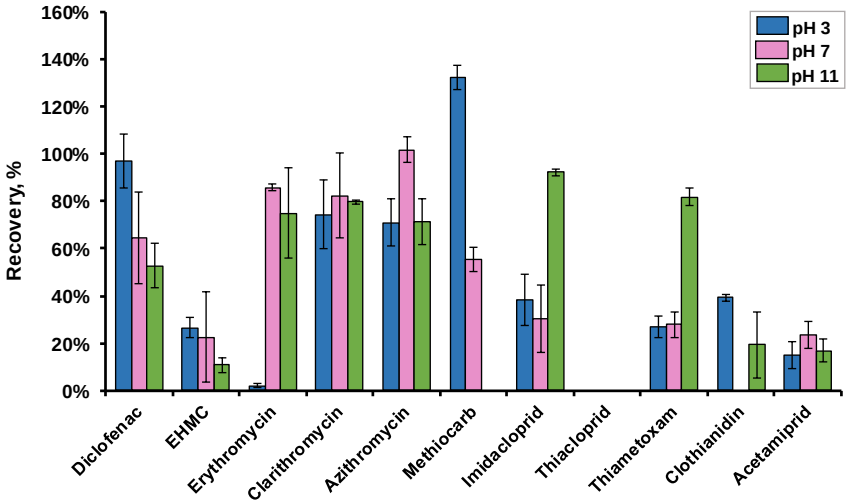


Figure 14. Recoveries obtained for Watch List compounds for different pH (3, 7 and 11), extracting 250 mL of surface water samples through Oasis® HLB cartridges and using methanol as solvent.

Figure 14 shows that the acidic pH led to higher recoveries for diclofenac, EHMC, methiocarb and clothianidin. However, in the case of EHMC, the recovery obtained at pH 7 was quite similar. Acetamiprid and the macrolide antibiotics had similar recoveries for the tested pH values, except erythromycin which was poorly recovered at pH 3. Although the basic pH would be the best option to extract imidacloprid and thiamethoxam, the overall results showed that pH 3 was appropriate for the majority of neonicotinoids [4]. Thereby, taking into account the reproducibility of the recoveries, the tandem MS signals of the compounds with lower recoveries and the compromise to include as much Watch List compounds as possible, the selected sample pH was 3.

4.2.3. Extraction solvent

The extraction solvent optimization was based on three different organic solvents (ethanol, methanol and acetonitrile). This step was performed by loading 250 mL of surface water sample through Oasis® HLB, previously acidified to pH 3. The results are shown in Figure 15.

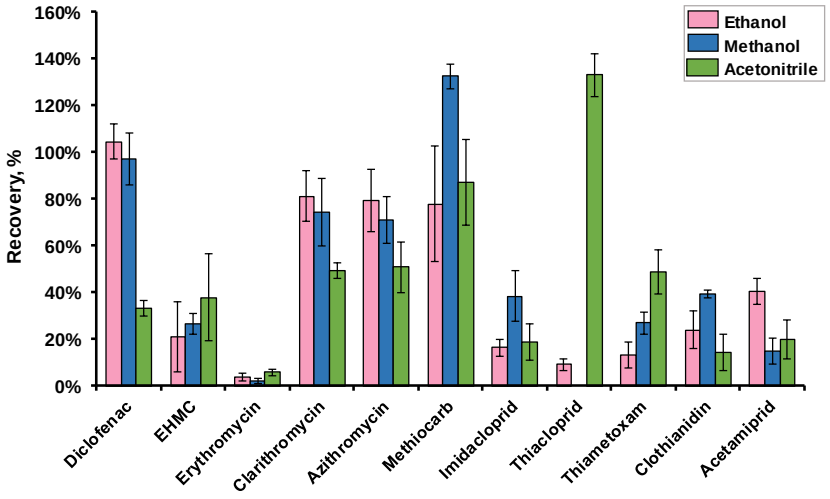


Figure 15. Recoveries obtained for Watch List compounds for different solvents (ethanol, methanol and acetonitrile), extracting 250 mL of surface water samples (pH 3) through Oasis® HLB cartridges.

It was possible to conclude that acetonitrile improved the recovery for 3 compounds, namely EHMC, erythromycin, thiacloprid and thiamethoxam (Figure 15). Methanol, the most widely used organic solvent on SPE procedures [4, 74, 114], was compared with ethanol that allowed to obtain recoveries slightly higher than methanol for most of the Watch List compounds. The compounds for which methanol gave significantly better recovery values than ethanol were methiocarb, imidacloprid and clothianidin. Moreover, ethanol is considered an eco-friendly solvent, minimizing the environmental impact resulting from the use of organic solvents, which allowed to comply the guidelines of green analytical chemistry [72]. Thus, the SPE procedure using ethanol as conditioning and eluting solvent is undoubtedly a great advance comparing to most methods using other solvents [72, 74].

4.2.4. Sample volume

The higher volume that allows to achieve the higher extraction efficiency and from which extraction efficiency declined is so-called breakthrough volume. The optimization of sample volume is a needful step to avoid overloading the SPE cartridge [90]. Different sample volumes (100, 250, 500 and 1000 mL) were tested and the results are shown in Figure 16.

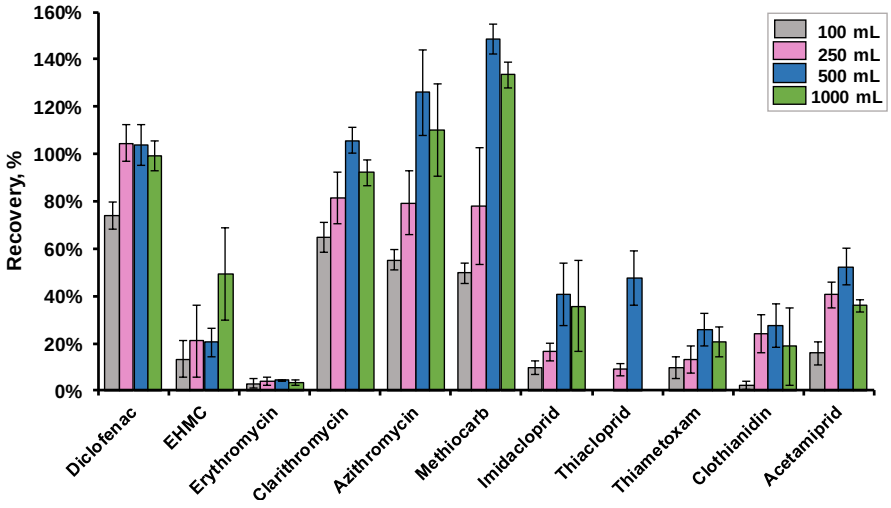


Figure 16. Recoveries obtained for Watch List compounds, extracting different sample volumes (100, 250, 500 e 1000 mL), of surface water samples (pH 3) through Oasis® HLB cartridges, using ethanol as solvent.

Except for EHMC, the maximum extraction efficiency was obtained for 500 mL of sample (Figure 16), the breakthrough volume, above which the extraction efficiency declined, such as previously observed by Ribeiro et al. [115] and Tan et al. [90]. Thus, 500 mL of sample volume was selected for method validation.

4.3. Method validation

The SPE-UHPLC-MS/MS method was validated based on international criteria [116] and published works [72, 115, 117, 118]. Recovery, accuracy, intra and inter-batch precision values are shown in Table 6. The linearity, range, instrument and method detection and quantification limits for each target analyte are shown in Table 5.

Table 5. Retention time, range, linearity, instrument and method detection and quantification limits for each target analyte.

Group	Analyte	Retention time (min)	Range (ng L^{-1})	r^2	IDL[a] (µg L^{-1})	IQL[b] (µg L^{-1})	MDL[c] (ng L^{-1})	MQL[d] (ng L^{-1})
Anti-inflammatory	Diclofenac	3.33	0.90–300	0.997	0.59	1.80	0.30	0.90
Organic UV Filter	2-Ethylhexyl 4-methoxycinnamate	4.28	8.08–100	0.995	5.33	16.16	2.67	8.08
Macrolide Antibiotics	Erythromycin	4.60	0.51–300	0.996	0.34	1.02	0.17	0.51
	Clarithromycin	5.25	0.03–100	0.994	0.02	0.07	0.01	0.03
	Azithromycin	5.30	0.37–100	0.997	0.24	0.73	0.12	0.37
Pesticide	Methiocarb	2.51	0.54–100	0.997	0.35	1.07	0.18	0.54
Neonicotinoids	Imidacloprid	1.72	6.06–400	0.996	4.00	12.12	2.00	6.06
	Thiacloprid	1.72	0.08–400	0.999	0.05	0.16	0.03	0.08
	Thiamethoxam	1.65	3.71–400	0.998	2.45	7.42	1.22	3.71
	Clothianidin	1.71	0.73–400	0.998	0.48	1.47	0.24	0.73
	Acetamiprid	1.69	0.98–75	0.996	0.64	1.95	0.32	0.98

[a] IDL, instrument detection limit; [b] IQL, instrument quantification limit.; [c] MDL, method detection limit; [d] MQL, method quantification limit.

The recoveries of the Watch List compounds studied in this work were compared, after pre-concentration of spiked blank samples at 300 ng L^{-1}, using the optimized SPE procedure, i.e., Oasis HLB cartridges loaded with 500 mL of water sample acidified (pH 3), conditioned and eluted with ethanol. Watch List compounds found in the surface water matrix were subtracted for recovery rate evaluation. The recoveries evaluated for surface water matrix were between 4.53 (± 0.16) % and 148.64 (± 6.47) % (Table 6). In general, EHMC, erythromycin and neonicotinoids were less recovered in all SPE procedures. The diverse recoveries are owing to the wide chemistry nature of the compounds; however, the reproducibility of the results and the use of matrix match calibration curves and internal calibration method allowed to pursue the method development. Three quality control standard extracts were used to assess the accuracy and intra and inter-batch precision. The accuracy ranged from 80.09% to 118.99% (Table 6), which is within the range of ± 20% of the nominal concentration, according to the international guidelines (80–120%) [116] and published works [117]. Regarding precision, relative standard deviation (RSD) of the replicate analyses was assessed (Table 6). Intra-batch precision values were below 13.13% and inter-batch precision under 13.84%, thus meeting the international criteria which suggest an agreement of the results traduced by a RSD lower than 15% between the different QC of the same concentration. [116]. The calibration curves (Table 5) were performed using the internal calibration method, by spiking the samples with isotopically labelled internal standards before SPE extraction. Different groups of target analytes were defined and one internal standard was set for each group of compounds (Table 4), according to other published works dealing with multi-class determination [59, 62]. The injection of the reconstituted ethanolic extracts gave correlation coefficients between 0.994 and 0.999 in the range of linearity (Table 5).

The method detection limits were between 0.01 and 2.67 ng L^{-1}, well below the "maximum acceptable method detection limits" recommended by Decision 495/2015/EU [29], which are within the range of 9 ─ 6000 ng L^{-1} for the target analytes. The method quantification limits were between 0.03 and 8.08 ng L^{-1}, providing to detect the Watch List compounds at trace concentrations adequate for the purpose of the method and according to the suggested by Decision 495/2015/EU [29].

Table 6. Recovery, accuracy and precision (intra- and inter-batch) for each target analyte.

Group	Analyte	Recovery (%)	Accuracy (%)	Intra-batch precision RSD (%)	Inter-batch precision RSD (%)
Anti-inflammatory	Diclofenac	103.93 ± 8.70	87.19─115.91	< 7.08	< 12.12
Organic UV Filter	2-Ethylhexyl 4-methoxycinnamate	20.42 ± 6.17	91.47─118.99	< 6.27	< 7.63
Macrolide Antibiotics	Erythromycin	4.53 ± 0.16	95.40─114.74	< 16.68	< 17.83
	Clarithromycin	105.62 ± 5.43	80.61─111.55	< 17.66	< 16.91
	Azithromycin	126.08 ± 18.03	80.09─117.04	< 10.16	< 7.11
Pesticide	Methiocarb	148.64 ± 6.47	83.81─115.08	< 8.79	< 8.81
Neonicotinoids	Imidacloprid	40.91 ± 13.18	84.72─109.43	< 16.68	< 12.46
	Thiacloprid	47.45 ± 11.50	88.47─90.47	< 11.16	< 11.46
	Thiamethoxam	25.90 ± 6.83	85.14─93.42	< 7.74	< 8.92
	Clothianidin	27.54 ± 9.42	87.48─87.79	< 9.45	< 8.23
	Acetamiprid	52.44 ± 7.77	99.29─116.10	< 10.87	< 9.22

[a] IDL, instrument detection limit; [b] IQL, instrument quantification limit.; [c] MDL, method detection limit; [d] MQL, method quantification limit.

4.4. Matrix effects

In the ionization source of the mass spectrometer, the ionization of target analytes can be reduced or enhanced, depending on the matrix effect, i.e., the influence of the matrix in the ionization process. The post-extraction addition method allows to assess this effect, consisting in the comparison of the signal given by chromatograms of SPE extracts of blank samples spiked with a solution containing the target compounds (post-spiked blank extracts) after subtracting the blank signal, with chromatograms of the standard solution with the theoretical concentration of the extracts. This ratio varied between 4.06 and 108.07%. Most compounds presented matrix effect < 100%, i.e., signal suppression, such as diclofenac, erythromycin, methiocarb, imidacloprid, imidacloprid, thiacloprid, thiamethoxam, clothianidin and acetamiprid. EHMC and azithromycin had matrix effect > 100%, i.e., presented a slight signal enhancement. Clarithromycin was the only compound almost not affected by surface water matrix effects.

4.5. Case of study: Sousa and Ave Rivers

4.5.1. Quantification of CECs

As referred above, surface water samples from various sampling points from Sousa River and Ave River were collected at 18th May 2016 and 1st June 2016, respectively. Site pictures can be found in Appendix D. From source until the mouth of rivers, 15 samples were collected from each river and analysed using the optimized SPE-UHPLC-MS/MS method above mentioned. The flow rate of Sousa River was determined (3.35 – 45.38 $m^3 s^{-1}$) and it was possible to relate the higher flow rates with the presence of tributaries, a waterfall or micro-hydric plant (Figure 17). It is important to notice that sampling point P11 is much less influenced by the river flows because it is near a micro-hydric plant and, thus, residence time is higher comparatively to the other sampling points. On the mouth of Sousa River, the flow rate decreased probably due to the influence of Douro River. The flow rate of Ave River was determined from sampling point P4 and varied between 13.68 and 86.67 $m^3 s^{-1}$; however, the interference of many dams/micro-hydric plants did not allow the continuous representation of this parameter (Figure 18). The flow rate of the Ave River achieved higher values than those of Sousa River.

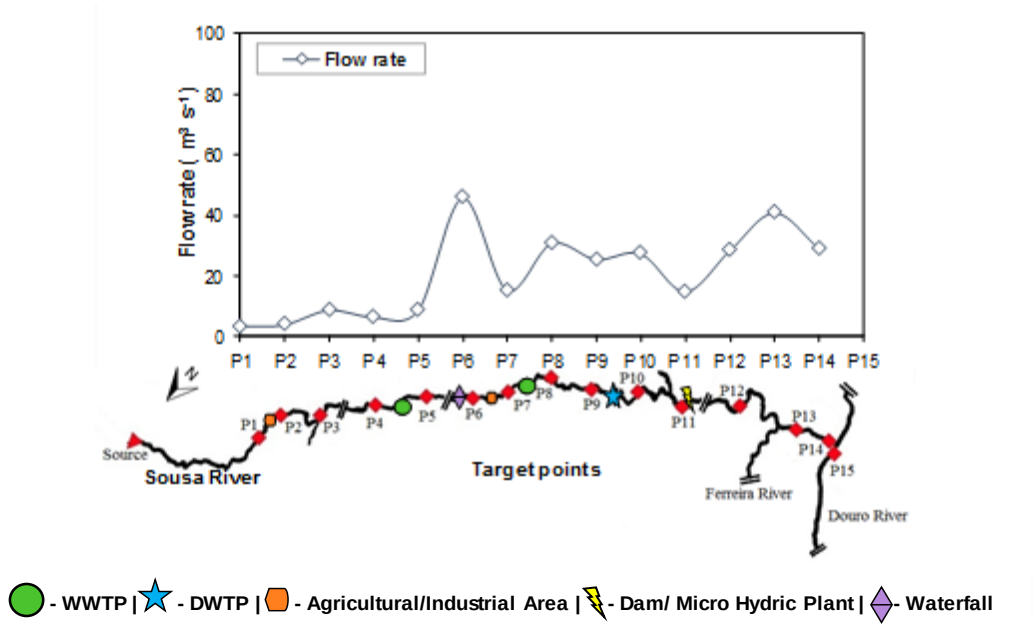


Figure 17. Flow rate (m^3s^{-1}) determined in the Sousa River.

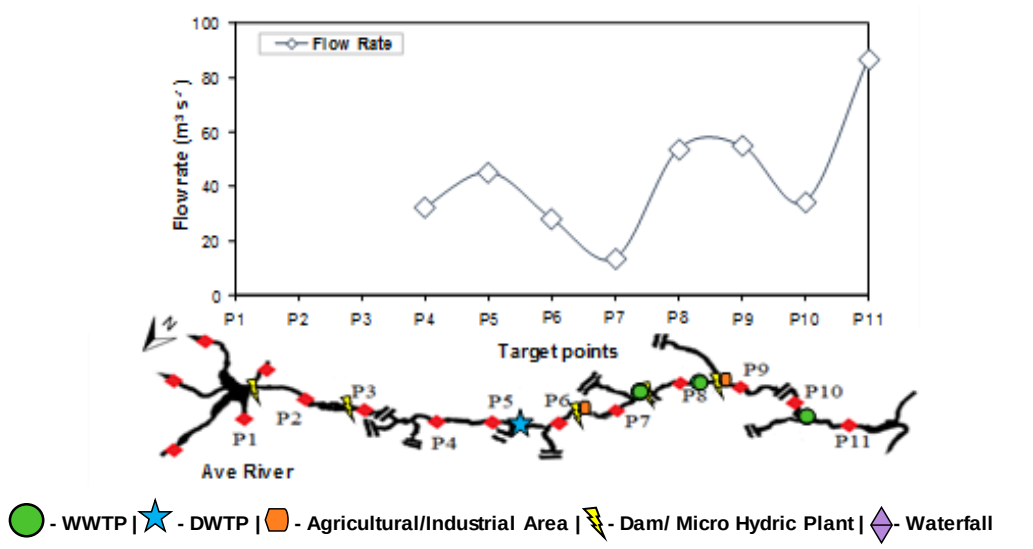


Figure 18. Flow rate (m^3s^{-1}) determined in the Ave River.

Figures 19 and 20 show the variation of concentrations (ng L^{-1}) of Watch List compounds found in Sousa and Ave Rivers, respectively. The compounds were determined by using the analytical methodology developed, described in Section 4.1 (UHPLC-MS/MS).

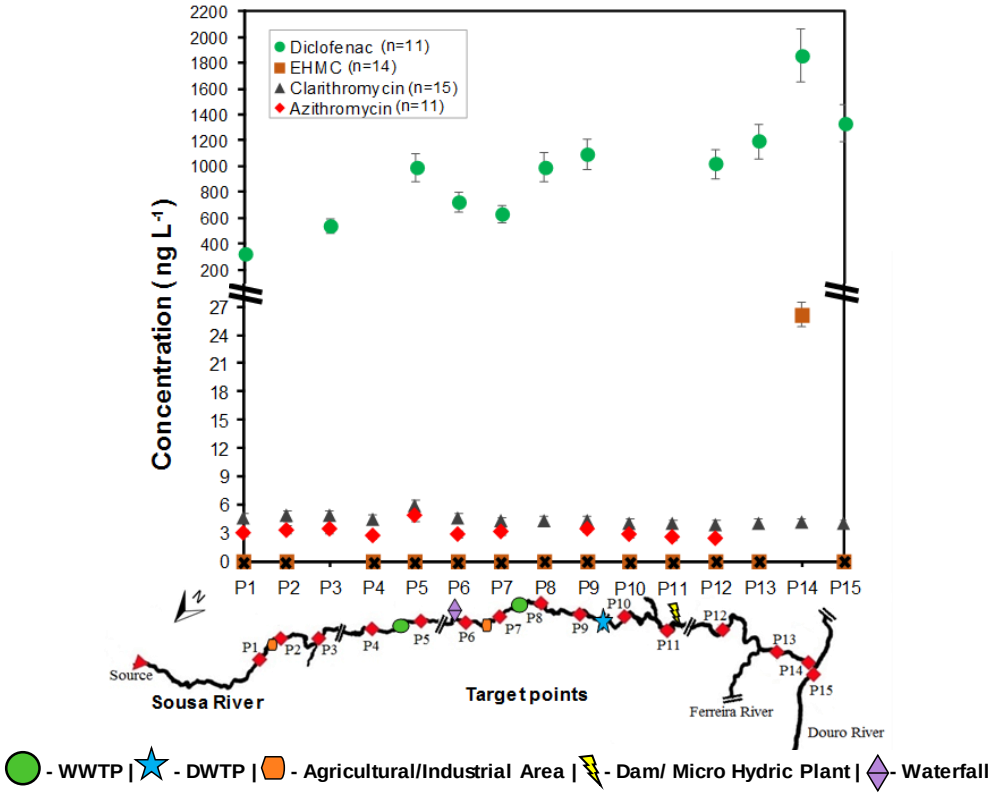


Figure 19. Variation of concentrations (ng L^{-1}) of Watch List compounds found in Sousa River (data points with × correspond to < MQL values).

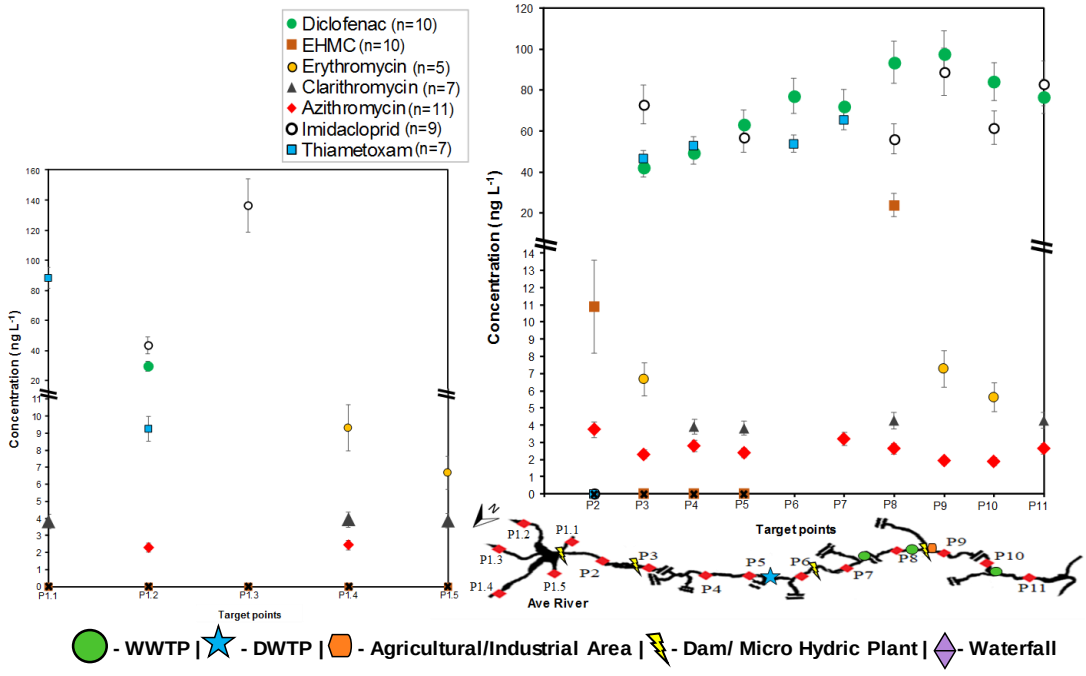


Figure 20. Variation of concentrations (ng L^{-1}) of Watch List compounds found in Ave River (data points with × correspond to < MQL values).

Four Watch List compounds were detected in Sousa River samples, namely diclofenac, EHMC clarithromycin and azithromycin. Clarithromycin (3.88 – 5.91 ng L^{-1}, n = 15) and EHMC (26.11 ng L^{-1} in one sample, all others were < MQL, n = 14) were the most frequently found CECs in

Sousa River, whereas diclofenac was quantified in 11 samples, but at much higher concentrations (up to 1855.95 ng L^{-1}). Azithromycin was found between 2.42 and 4.83 ng L^{-1}, also in 11 samples. Seven CECs were found in Ave River samples. The most frequent micropollutants were clarithromycin, EHMC and azithromycin. Diclofenac presented a concentration between 29.53 and 97.95 ng L^{-1}, well below the concentrations determined for Sousa River. EHMC was detected up to 23.86 ng L^{-1}. Clarithromycin (3.81 – 5.28 ng L^{-1}) and azithromycin (1.91 – 3.73 ng L^{-1}) were found at similar concentrations than those determined in Sousa River. However, for Ave River, one of the most industrialized rivers of Portugal [84], other 3 compounds were detected, erythromycin (5.62 – 9.30 ng L^{-1}) and two neonicotinoids, imidacloprid (up to 136.52 ng L^{-1}) and thiamethoxam (up to 88.34 ng L^{-1}).

4.5.2. Physical-chemical parameters

- **Sousa River**

In order to evaluate water quality, physical-chemical parameters were also determined at each sampling point. Results obtained for all parameters are shown in Tables E1 and E2– Appendix E.

Physical-chemical parameters were also analysed, such as pH, DOC, temperature, dissolved oxygen, conductivity, salinity, oxidation-reduction potential, total dissolved solids, turbidity and flow rate. Although DOC concentrations were generally constant (Figure 21), they increased at sampling points P3, P11, P13 and P15, showing the influence of tributaries on DOC. Figure 21 also illustrates that pH values were quite similar along the river, ranging between 6.34 and 7.35.

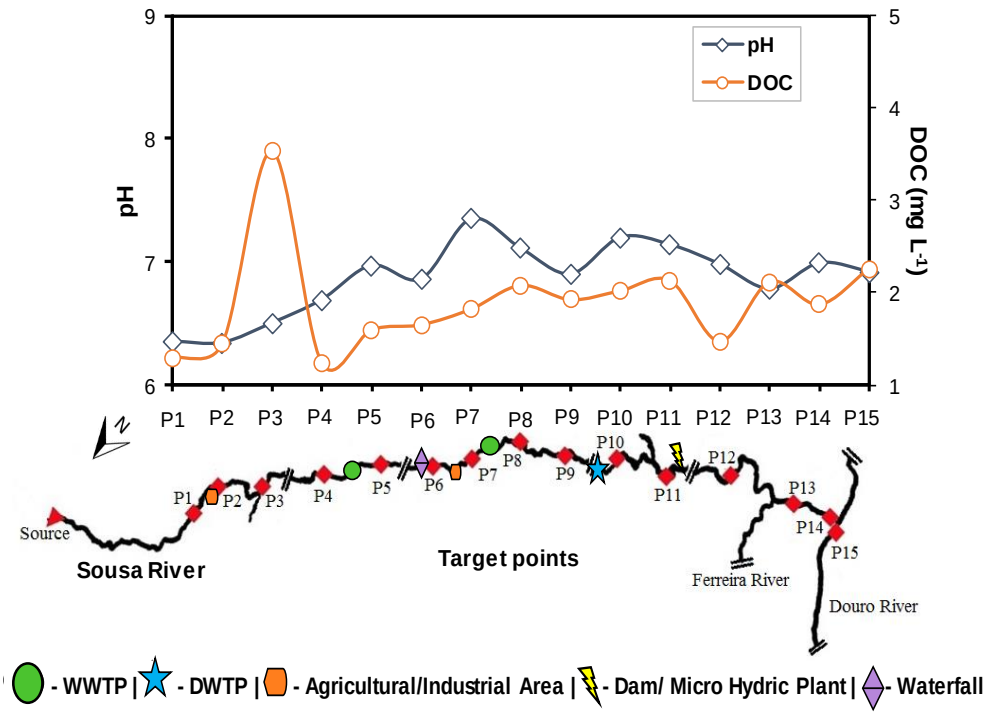


Figure 21. pH values and DOC concentrations (mg L^{-1}) in Sousa River.

Temperature tended to increase from the source to the mouth of the River from 13.9 to 17.3 ºC, as expected due to the increase of the ambient temperature along the sampling day, which started at the source (in the beginning of the morning) and ended at the mouth (at the end of the afternoon). Dissolved oxygen concentration was almost constant along the River, varying between 9.05 and 10.92 mg L^{-1} (Figure 22), exceptions being found at the mouth of the river.

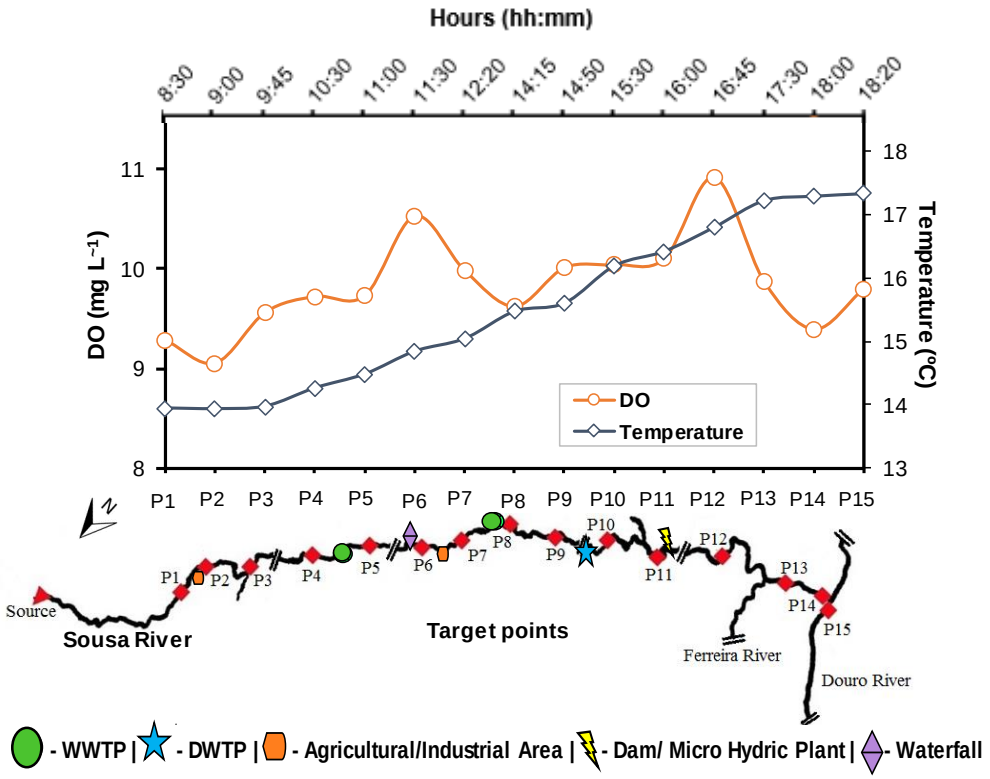


Figure 22. Temperature values and DO concentrations (mg L^{-1}) in Sousa River.

Along the River, conductivity varied slightly (179 – 202 µS cm^{-1}), while salinity did not change (0.08 – 0.1 Practical Salinity Units (PSU)). Oxidation-reduction potential was measured between 214.4 and 264.4 mV. Total dissolved solids were found between 89 and 103 mg L^{-1} and turbidity varied between 1.40 and 8.40 Nephelometric Turbidity Units (NTU). All results (Table E1) comply with values settled by the Portuguese law Decreto-Lei nº 236/98, which defines the quality standards for superficial and ground waters intended to be employed for the production of water for human use.

Ions concentration were also analysed (Table E2). The variation of cations along the River was not significant, and sampling points P5 and P8 (located after WWTPs) presented a very slight increase in the concentration of sodium (Figure 23), with ammonium only detected at these sampling points under their limit of quantification (data not shown). Regarding anions, chloride concentration was incremented at the above mentioned points. Bromate was not detected and bromide and nitrate were only detected under their limits of quantification. Furthermore, after Ferreira River tributary (P13) anions concentration significantly increased due the influence of this River and the Douro River (Figure 24).

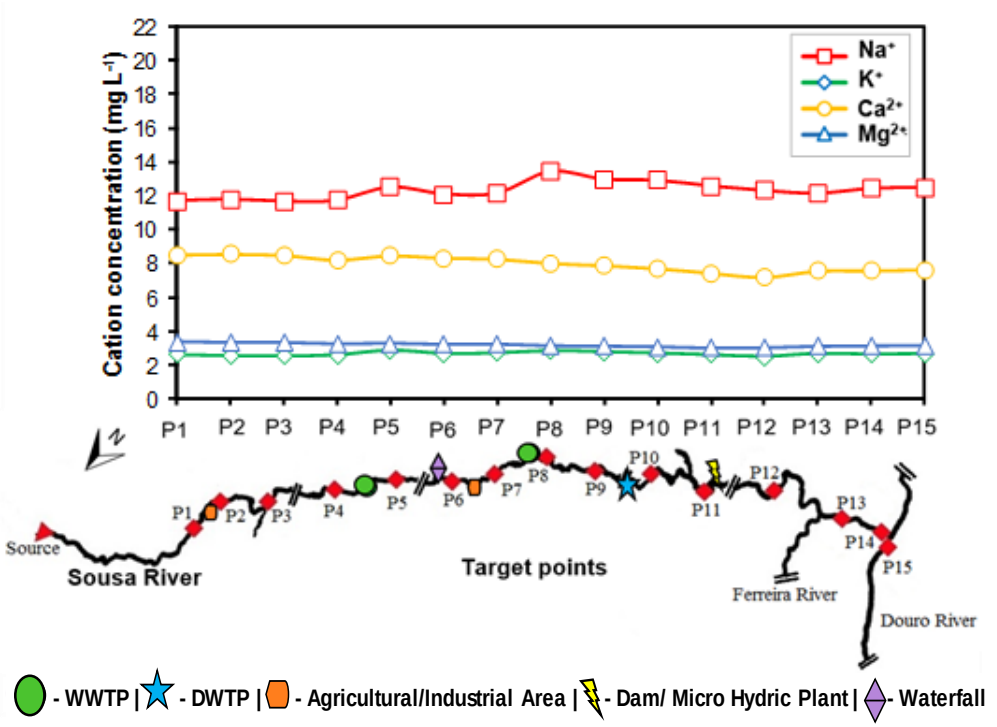


Figure 23. Sodium, potassium, calcium and magnesium in Sousa River.

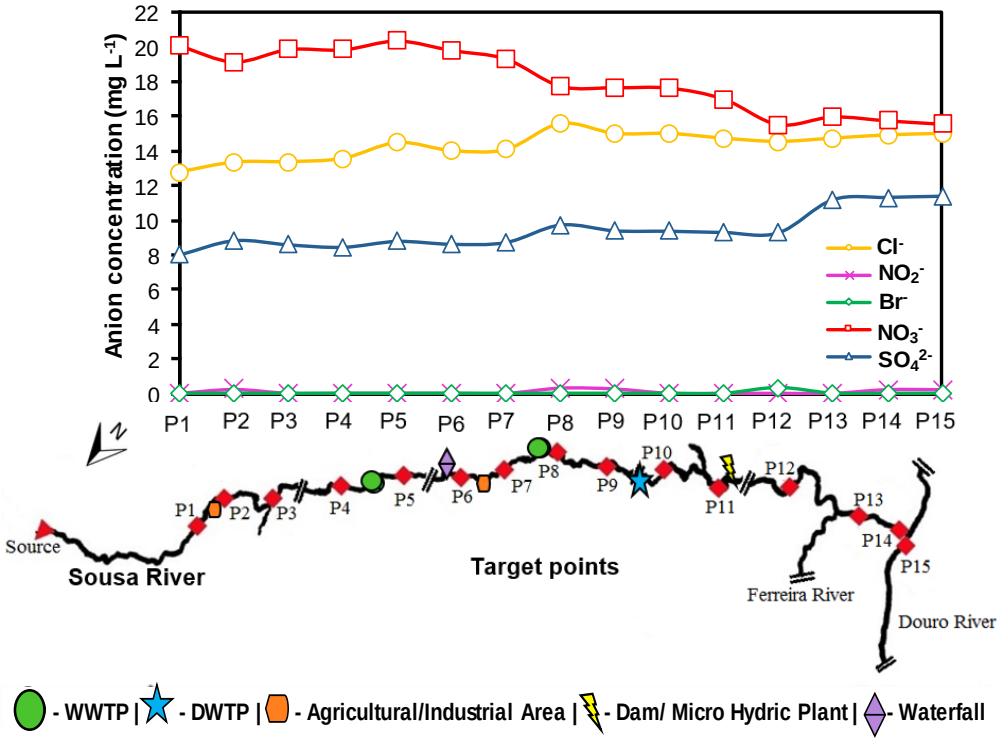


Figure 24. Chloride, nitrite, bromide, nitrate and sulfate concentrations (mg L^{-1}) in Sousa River.

▪ Ave River

Regarding the Ave River, Figure 25 shows that DOC concentration increased after P5, and after sampling point P8. It was also possible to observe the influence of tributaries/effluents, as in the case of the Sousa River. Unlike to Sousa River, pH values increased along Ave River, from 6.25 up to 8.45, from Ermal dam until sampling point P9, after which the pH decreased until 7.06 in the estuary. The higher value of pH was measured in sampling point P9, i.e. after Burgães WWTP and Vizela River. These results were expected considering the large amount of textile dyeing companies located at the Ave valley, producing basic effluents.

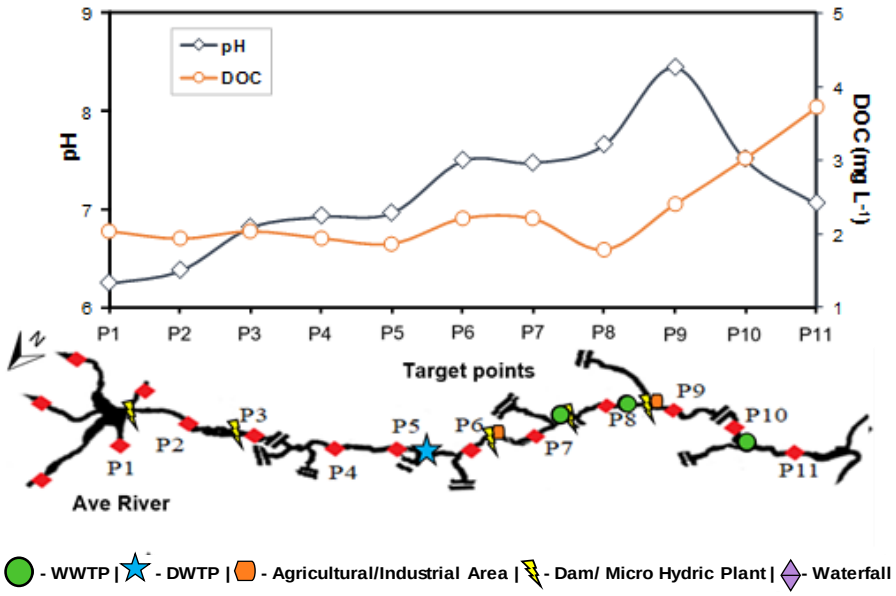


Figure 25. pH values and DOC concentrations (mg L^{-1}) in Ave River.

Figure 26 shows that the temperature increased along of the Ave River, after P2 (14.15 – 19.77 ºC), once again probably due to the time of the day at which the samples were collected. The dissolved oxygen concentration varied between 9.05 and 10.81 mg L^{-1}. Sampling point P1 was an exception, with a high temperature and low dissolved oxygen concentration, in comparison with the downstream sampling points, since it is much less influenced by the river flows because it is located at a dam and, thus, residence time is significantly higher in comparison to the other sampling points.

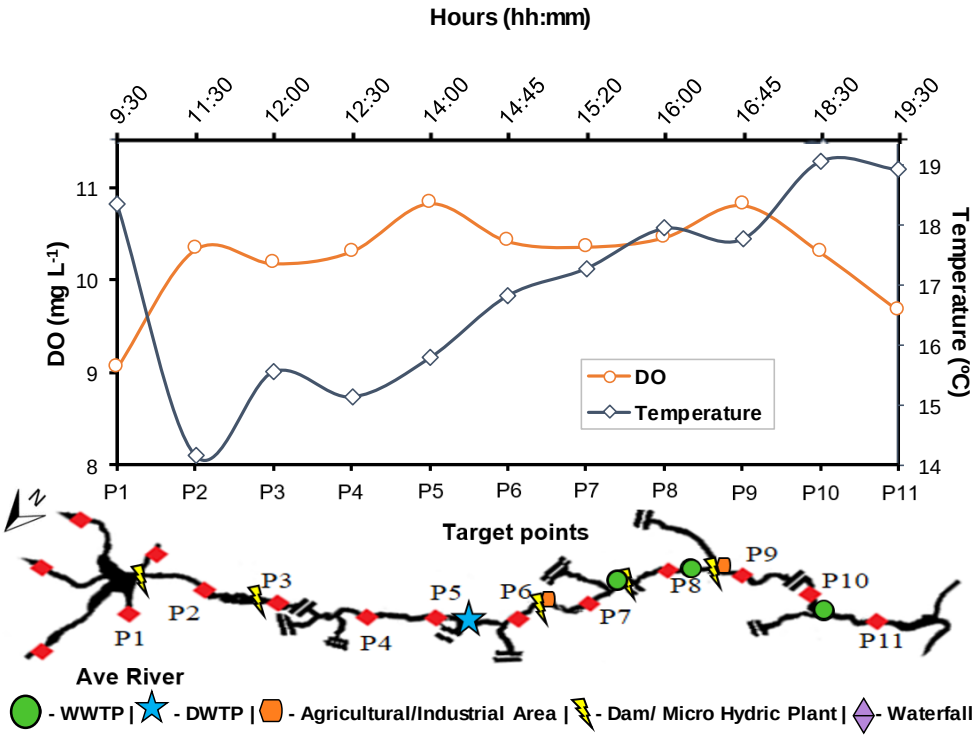


Figure 26. Temperature values and DO concentrations (mg L^{-1}) in Ave River.

Along the Ave River, the conductivity was measured between 66 and 2375 µS cm^{-1} (Table E3), with values 10 fold higher than those determined in the Sousa River, again explained by the influence of the effluents from the textile companies located at the Ave valley. Salinity varied from 0.03 to 1.23 PSU. A gradient of salinity and conductivity was observed from the sampling point P1 (lower values) to the river mouth P11 (higher values). Oxidation-reduction potential presented results between 187.1 and 280.8 mV. Total dissolved solids were found at concentrations between 33 and 1190 mg L^{-1}, well above the maximum value found in Sousa River. Turbidity almost did not vary. The levels of phosphates and chlorides for P10 and P11, respectively, were above the maximum values settled by the Portuguese law Decreto-Lei nº 236/98 (up to 0.4 mg L^{-1} for phosphates and 200 mg L^{-1} for chlorides). Conductivity values were also above the maximum values from sampling point P8. Related data may be consulted in Appendix E.

Some studied cations were detected in Ave River, such as sodium, potassium, calcium and magnesium and anions, such as chloride, nitrate and sulfate (Figure 27). Ammonium, bromate and phosphate were not detected. Bromide was detected at low concentrations only at sampling point P11 (Figure 28).

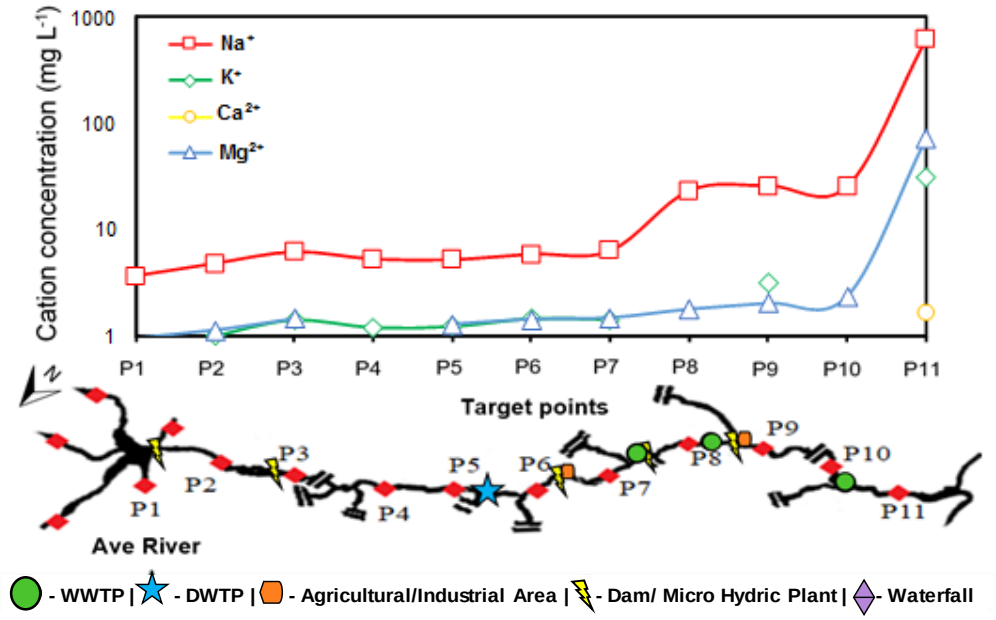


Figure 27. Sodium, potassium, calcium and magnesium concentrations (mg L^{-1}) in Ave River.

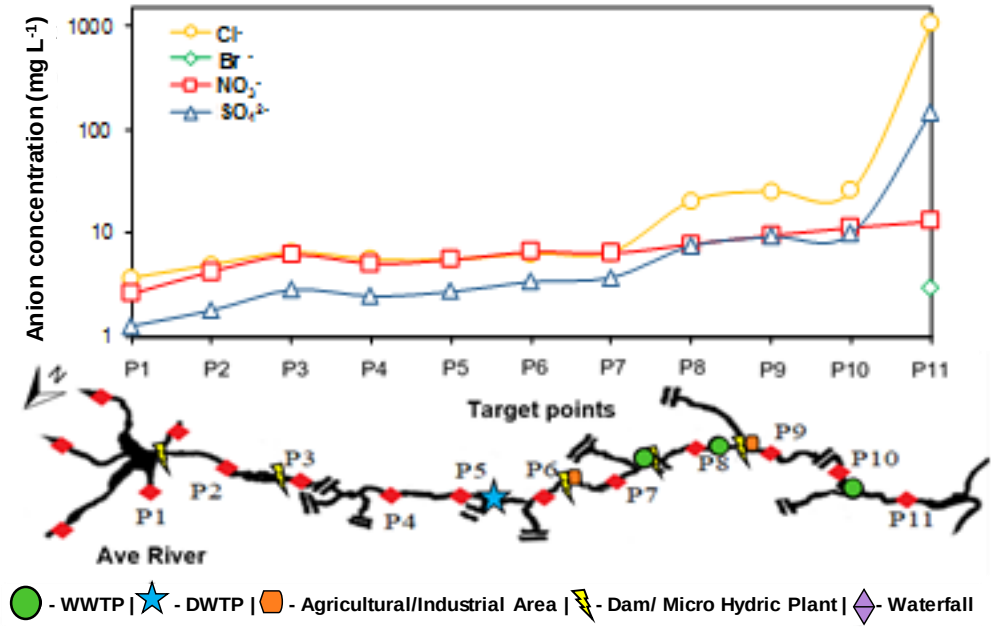


Figure 28. Chloride, bromide, nitrate and sulfate concentrations (mg L^{-1}) in Ave River.

In general, anions concentrations increased slightly before Guilhofrei dam (P3). After WWTPs of Serzedelo and Burgães and Vizela River (P8 and P9), ions concentration increased. Near the estuary, ions concentration presented higher values of sodium, magnesium, chloride and sulfate, in some cases by more than one order of magnitude, such as sodium and chloride, as expected due to the influence of the sea [119].

4.5.3. Comparison of CECs in Sousa and Ave Rivers

Figures 29 and 30 show the range of concentrations (ng L^{-1}) of Watch List compounds found in Sousa and Ave Rivers.

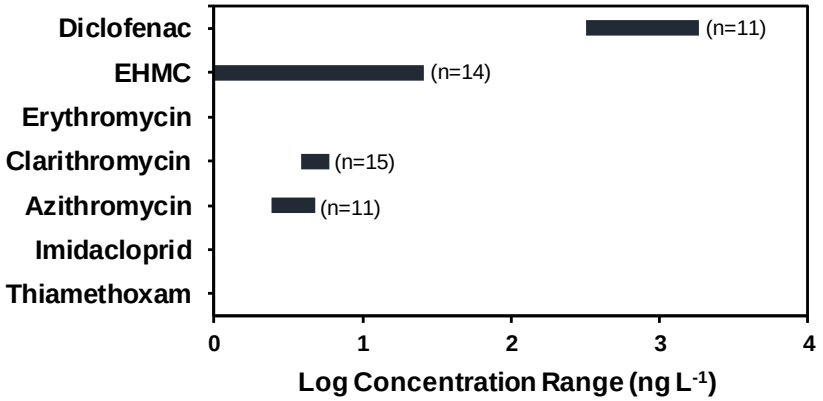


Figure 29. Range of concentrations (ng L^{-1}) of CECS found in Sousa River.

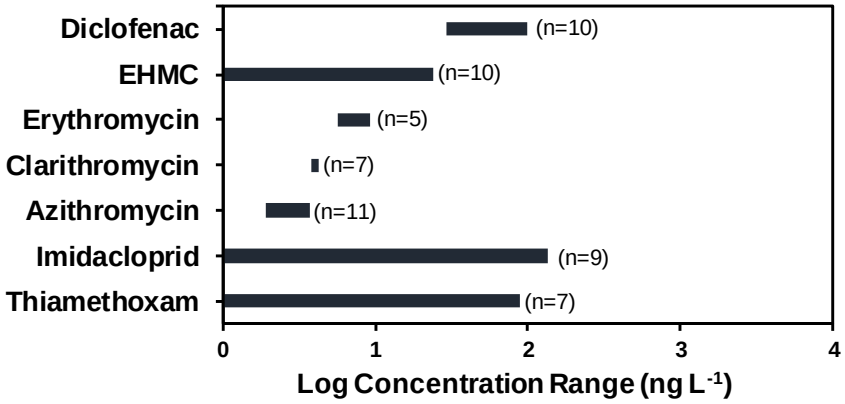


Figure 30. Range of concentrations (ng L^{-1}) of CECS found in Ave River.

From the 11 compounds that the analytical method allows to determine, 4 were detected and quantified in Sousa River samples and 7 were found in Ave River samples. The results are shown in Table F – Appendix F. The most frequent micropollutants were clarithromycin and EHMC in Sousa River and azithromycin in Ave River. Diclofenac presented a high concentration between 319.83 – 1855.95 ng L^{-1} and 29.53 – 97.95 ng L^{-1} for Sousa and Ave Rivers, respectively. EHMC was found at similar concentrations in the Sousa and Ave Rivers (up to 26.11 ng L^{-1} and up to 23.86 ng L^{-1}, respectively). Clarithromycin and azithromycin were found at low concentrations, respectively in the range of 3.88 – 5.91 ng L^{-1} and 2.42 – 4.83 ng L^{-1} in Sousa River and 3.81 – 5.28 ng L^{-1} and 1.91 – 3.73 ng L^{-1} in Ave River. For the Ave River, other 3 compounds were found, erythromycin (5.62 – 9.30 ng L^{-1}) and two neonicotinoids, imidacloprid (up to 136.52 ng L^{-1}) and thiamethoxam (up to 88.34 ng L^{-1}). The higher concentration of diclofenac as well as the

presence of macrolide antibiotics may be justified by the season in which the samples were taken, since these drugs are quite used to treat inflammatory and infectious respiratory diseases more common during winter and spring, and posteriorly discharged into the sewage system reaching surface waters with a poor removal by WWTPs. The range of concentrations found in this work are similar to those found in USA and South Africa [62, 105]; however, the comparison is difficult, since the activities, such as domestic and industrial activities, vary among different regions. Approximately, 90% of personal care products contain EHMC, generally with 0.5 – 10% in such type of products [120, 121]. Despite UV-filters are mainly used in cosmetics, they are also included in a wide range of products including adhesives, plastics, paint and rubber in order to protect from UV degradation [77]. Relatively to neonicotinoids found in the Ave River, the presence of agrochemical industries and intense agriculture/livestock areas may originate their presence in surface water. These results are similar to other studies reported by Sanchez-Bayo in Australia [66]. Thus, the presence of the Watch List compounds in surface water collected in these Rivers is probably related to their chemical properties, high consumption or use of products containing them and the inability of WWTPs to remove these CECs.

5. Conclusions

A sensitive multi-residual analytical method based on SPE followed by UHPLC–MS/MS was developed and optimized for simultaneous analysis in surface water of 11 Watch List compounds of EU Commission Decision 495/2015/EU, namely diclofenac, EHMC, macrolide antibiotics, methiocarb and neonicotinoids. The highest recoveries for the majority of target analytes in the SPE procedure were achieved using OASIS® HLB cartridges and ethanol as the elution solvent. Ethanol is considered an eco-friendly solvent, minimizing the environmental impact resulting from the use of organic solvents, allowing a compromise with the guidelines of green analytical chemistry. The optimal sample pH value for extraction of these compounds from surface water was 3, and the optimal water sample volume was 500 mL. Detection of all selected analytes was based on the analyses of the protonated molecule $[M+H]^+$, except in the case of diclofenac, which was based on the analysis of the deprotonated molecule $[M-H]^-$. All analytes produced more than one fragment ion, the most abundant was used for quantification and the additional fragment ions were used for confirmation. The SPE-UHPLC-MS/MS method validation was performed according to the international criteria and the results obtained for selectivity, linearity and range, MDL and MQL, accuracy, recovery and precision were in agreement with the guidelines. The developed method was applied in the analysis of 30 real surface water samples collected in Sousa and Ave Rivers. From the 11 studied compounds, 4 (diclofenac, EHMC, clarithromycin and azithromycin) were detected and quantified in the Sousa River samples and 7 (diclofenac, EHMC, erythromycin, clarithromycin, azithromycin, imidacloprid and thiamethoxam) were detected in the Ave River samples. The most frequently found micropollutants were clarithromycin and EHMC for Sousa River and azithromycin for Ave River. The higher concentrations detected were for diclofenac within the range 319.83 – 1855.95 ng L^{-1} in the Sousa River, and imidacloprid with a concentration up to 136.52 ng L^{-1} in the Ave River. Physical-chemical parameters, such as pH, temperature, dissolved oxygen, conductivity, salinity, oxidation-reduction potential, total dissolved solids, turbidity and flow rate were also analysed for both rivers. This work provides the first data on the identification and quantification of a wide range of Watch List compounds in surface water from two different rivers of Northern Portugal and shows the importance to extend the study of the CECs presence in surface water.

6. Future work

The development and validation of the SPE-UHPLC-MS/MS method in this work was an advantage for rivers monitoring, mainly for Watch List compounds. In this sense, some suggested future work can be addressed:

- To complete the development of the method in order to cover all Watch List compounds;
- To extend the SPE-UHPLC-MS/MS method for possible determination of by-products resulting from the degradation of Watch List compounds;
- To develop an optimized sampling campaign at different seasons, during 1 year, for collection and analysis of surface water samples of Sousa and Ave Rivers, and/or other Portuguese rivers;
- To develop a SPE-UHPLC-MS/MS method to monitor other CECs or PSs in surface water in order to enrich monitoring campaigns;
- To develop methodologies for determination of CECs in soils and sediments near to surface water.

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Appendix A: Watch list of substances for Union-wide monitoring in the field of water policy

Table A1. Watch list of substances for Union-wide monitoring in the field of water policy defined in the Commission Implementing Decision 2015/495/EU of March 2015 [29].

Name of substance/group of substances	CAS number ([1])	EU number ([2])	Indicative analytical method ([3])([4])([5])	Maximum acceptable method detection limit (ng l^{-1})
17-Alpha-ethinylestradiol (EE2)	57-63-6	200-342-2	Large-volume SPE — LC-MS-MS	0.035
17-Beta-estradiol (E2), Estrone (E1)	50-28-2, 53-16-7	200-023-8	SPE — LC-MS-MS	0.4
Diclofenac	15307-86-5	239-348-5	SPE — LC-MS-MS	10
2,6-Ditert-butyl-4-methylphenol	128-37-0	204-881-4	SPE — GC-MS	3160
2-Ethylhexyl 4-methoxycinnamate	5466-77-3	226-775-7	SPE — LC-MS-MS or GC-MS	6 000
Macrolide antibiotics ([6])			SPE — LC-MS-MS	90
Methiocarb	2032-65-7	217-991-2	SPE — LC-MS-MS or GC-MS	10
Neonicotinoids ([7])			SPE — LC-MS-MS	9
Oxadiazon	19666-30-9	243-215-7	LLE/SPE — GC-MS	88
Triallat	2303-17-5	218-962-7	LLE/SPE — GC-MS or LC-MS-MS	670

([1]) Chemical Abstracts Service.

([2]) European Union number — not available for all substances.

([3]) To ensure comparability of results from different Member States, all substances shall be monitored in whole water samples.

([4]) Extraction methods:

LLE—liquid-liquid extraction,

SPE—solid-phase extraction.

Analytical methods:

GC-MS—Gas chromatography-mass spectrometry,

LC-MS-MS—Liquid chromatography (tandem) triple quadrupole mass spectrometry.

([5]) For monitoring 2-Ethylhexyl 4-methoxycinnamate in suspended particulate matter (SPM) or in sediment (size < 63 μm), the following analytical method is indicated: SLE (solid liquid extraction) — GC-MS, with a maximum detection limit of 0.2 mg kg^{-1}.

([6]) Erythromycin (CAS number 114-07-8, EU number 204-040-1), Clarithromycin (CAS number 81103-11-9), Azithromycin (CAS number 83905-01-5, EU number 617-500-5).

([7]) Imidacloprid (CAS number 105827-78-9/138261-41-3, EU number 428-040-8), Thiacloprid (CAS number 111988-49-9), Thiamethoxam (CAS number 153719-23-4, EU number 428-650-4), Clothianidin (CAS number 210880-92-5, EU number 433-460-1), Acetamiprid (CAS number 135410-20-7/160430-64-8).

Appendix B: Mobile phase

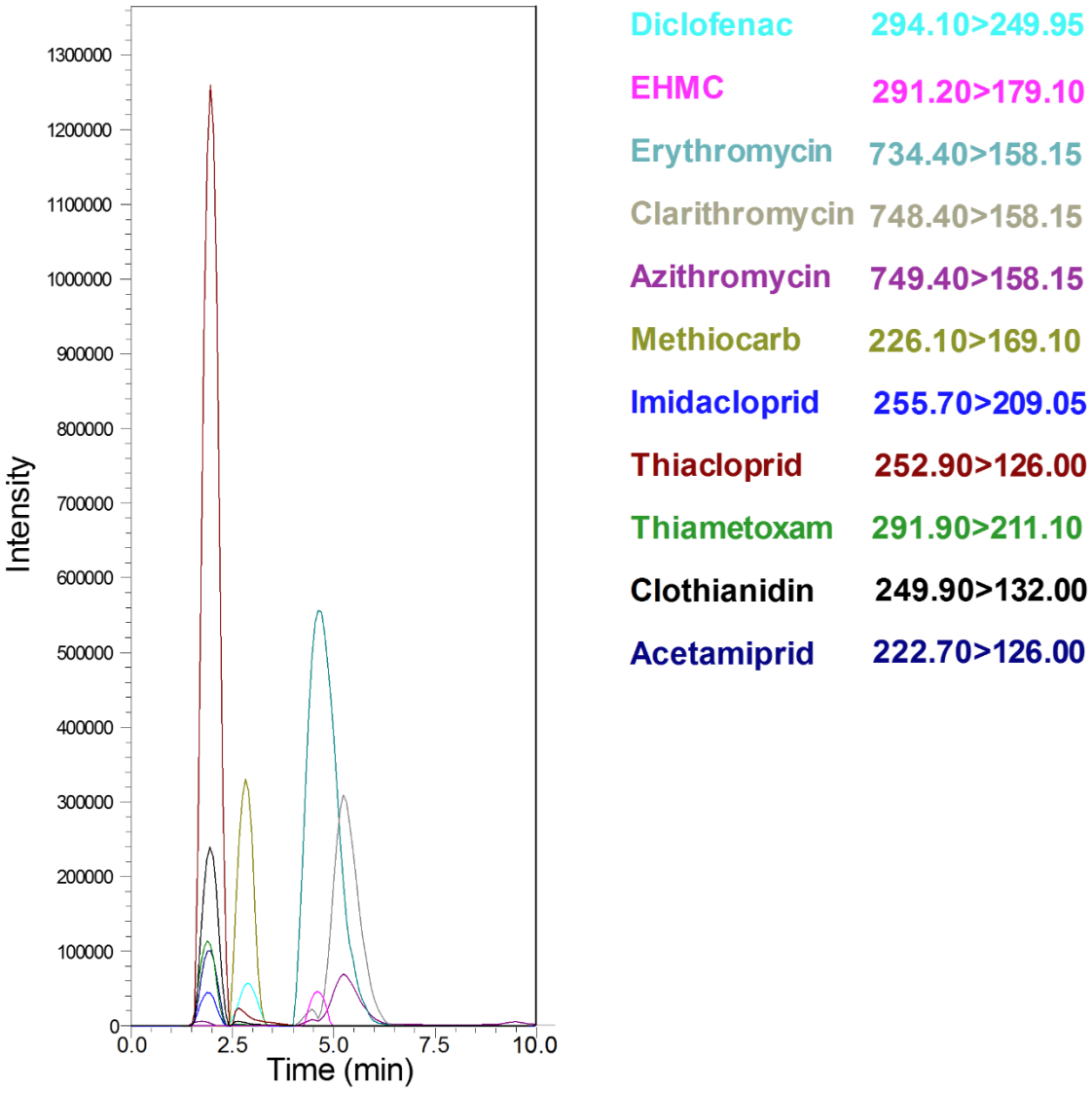

Figure B1. Chromatogram of the target analytes obtained with optimized mobile phase. Conditions: Kinetex™ 1.7 µm XB-C18 100 Å column (100 × 2.1 mm, i.d.), using a mobile phase of methanol/water (75/25, v/v) performed at gradient mode at a flow rate of 0.25 mL min^{-1}.

Appendix C: MS parameters

- **Nebulizing gas flow**

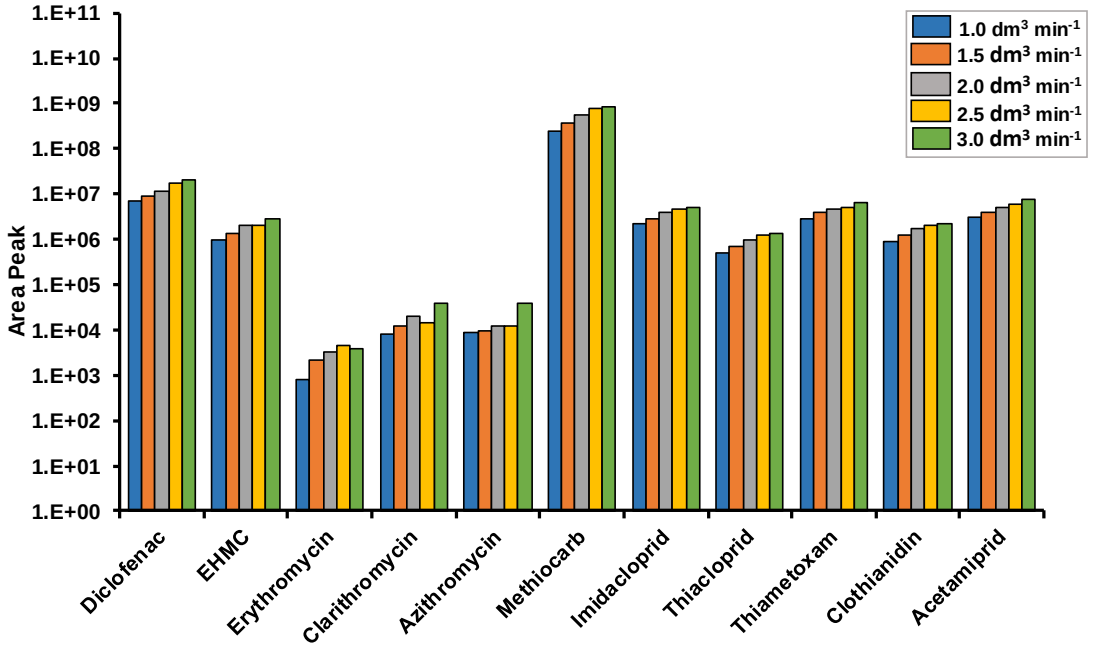

Figure C1. Results obtained for target analytes with different nebulizing gas flow values: 1.0, 1.5, 2.0, 2.5 and 3.0 dm^3 min^{-1}.

- **Drying gas flow**

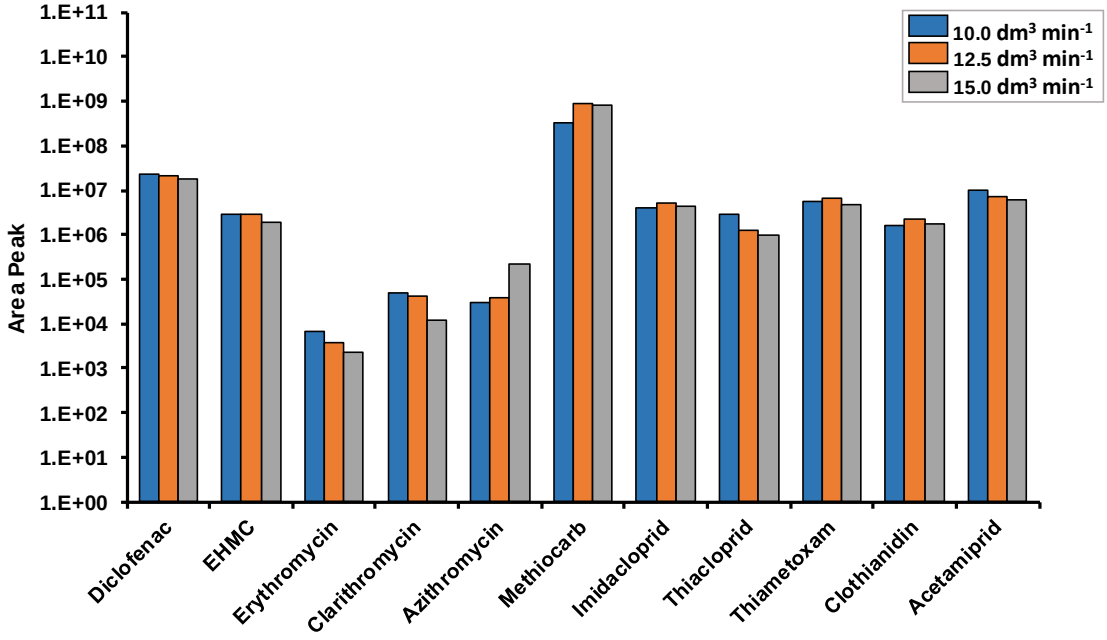

Figure C2. Results obtained for target analytes with different drying gas flow values: 10.0, 12.5 and 15.0 dm^3 min^{-1}.

- **Capillary voltage**

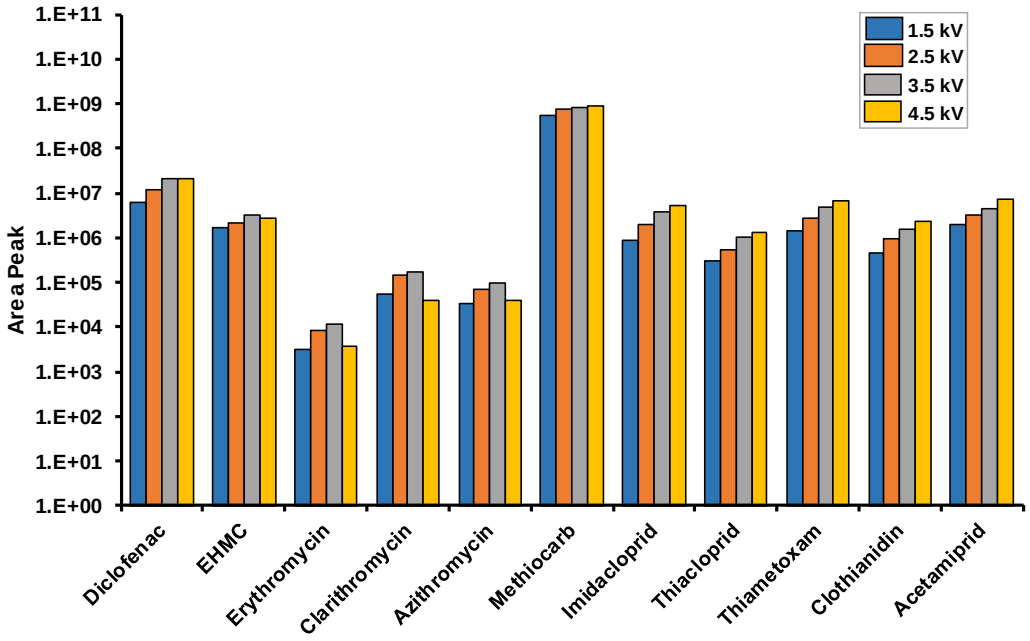

Figure C3. Results obtained for target analytes with different capillary voltage values: 1.5, 2.5, 3.5 and 4.5 kV.

- **Desolvation temperature**

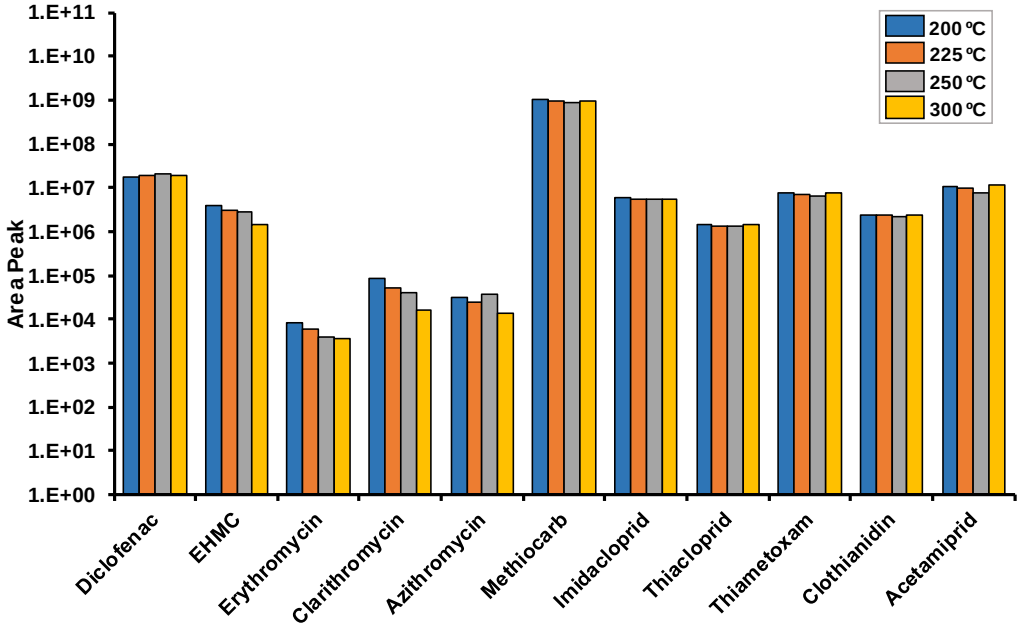

Figure C4. Results obtained for target analytes with different desolvation temperature values: 200, 225, 250 and 300 ºC.

- **Source temperature**

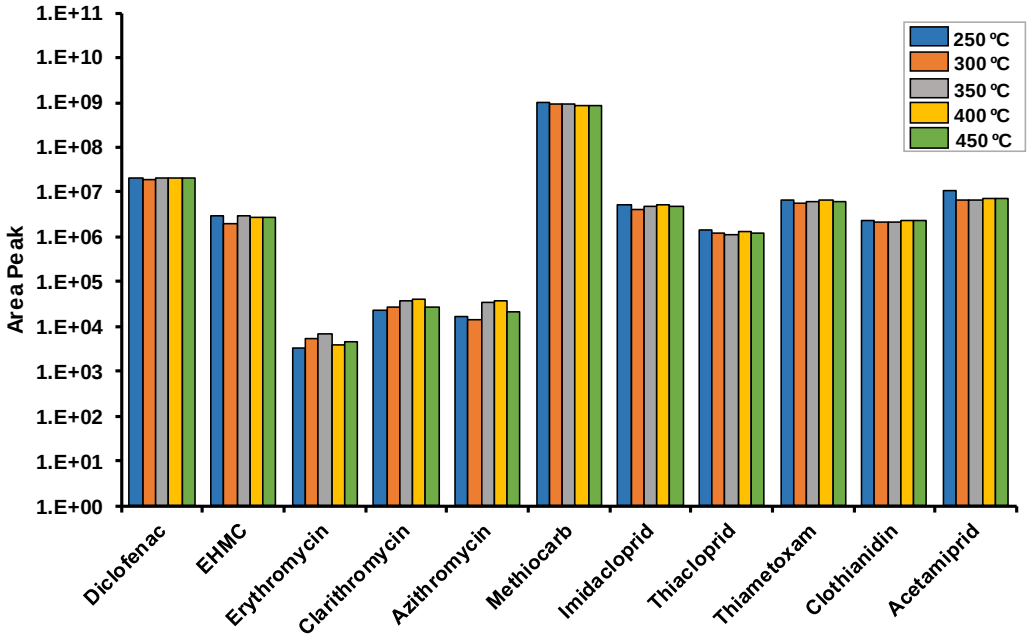

Figure C5. Results obtained for target analytes with different source temperature values: 200, 300, 350, 400 and 450 ºC.

Appendix D: Site pictures of the Sousa and Ave Rivers

Figure D1. Site pictures of the Sousa River.

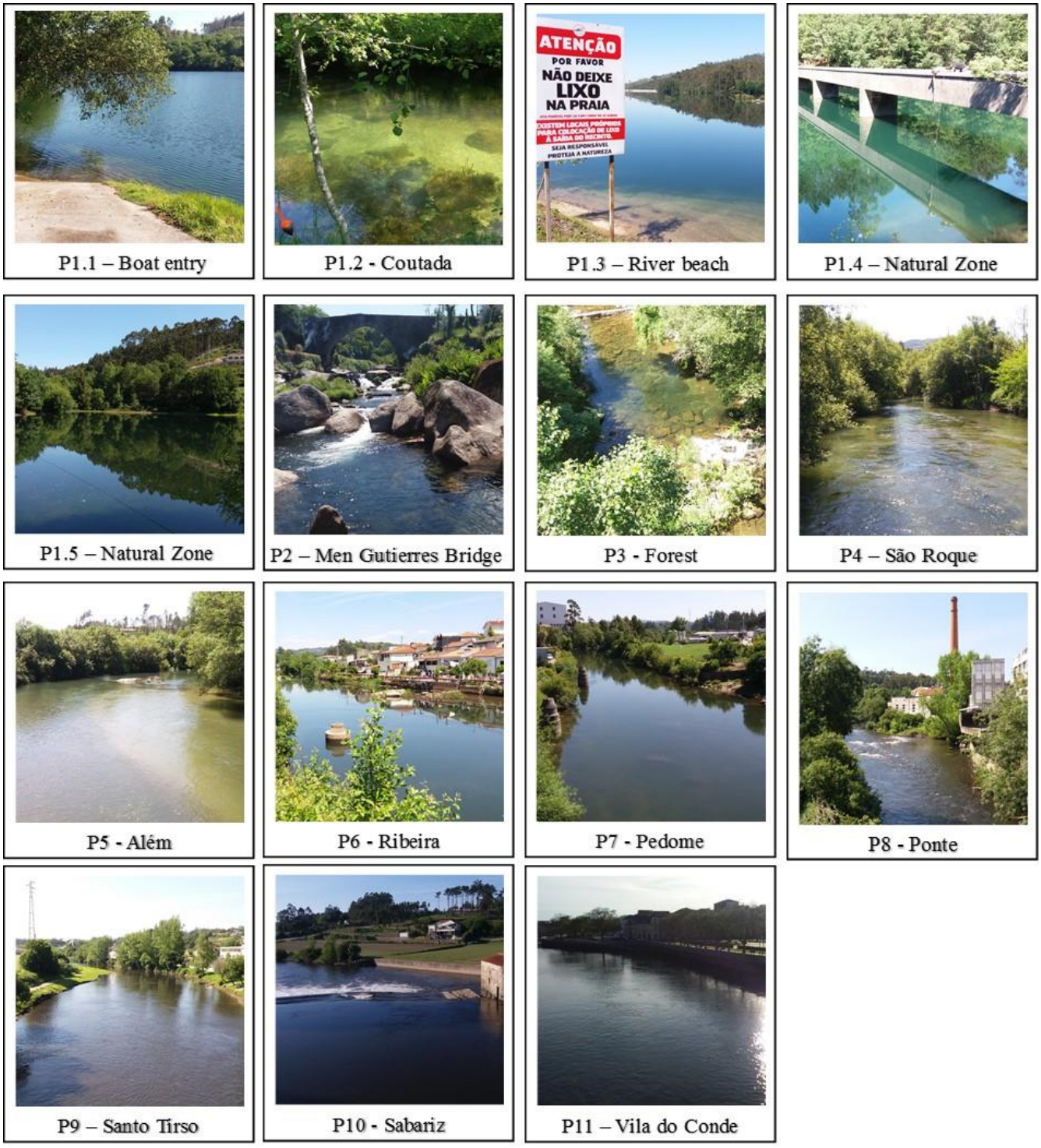

Figure D2. Site pictures of the Ave River.

Appendix E: Physical-chemical parameters

Table E1. Physical-chemical parameters measured in Sousa River.

Target Point	Characteristics / Location	Lat. (ºN)	Long (ºW)	pH	Temperature (ºC)	DO (mg L^{-1})	Conductivity (µS cm^{-1})	Salinity (PSU)	ORP (mV)	TDS (ppm)	Turbidity (NTU)	DOC (mg L^{-1})	TC (mg L^{-1})	IC (mg L^{-1})	Flow Rate (m^3 s^{-1})
S	Source	41.371785	-8.167365	-	-	-	-	-	-	-	-	1.65	6.62	4.97	-
1	São Cristovão Street, Lordelo	41.306724	-8.219165	6.35	13.94	9.28	202	0.1	214.4	101	4.60	1.30	6.17	4.87	3.35
2	Veiga Bridge	41.299408	-8.217599	6.34	13.93	9.05	203	0.1	217.3	101	5.50	1.45	6.51	5.06	3.89
3	Ponte Amial Street, Cernadelo	41.290617	-8.235088	6.50	13.97	9.56	201	0.1	227.0	99	8.40	3.53	8.05	4.52	8.73
4	Lodares (before WWTP)	41.238516	-8.277059	6.69	14.26	9.72	199	0.09	226.6	100	7.40	1.25	5.77	4.53	6.44
5	Novelas, Penafiel (after WWTP)	41.223998	-8.290396	6.97	14.48	9.73	206	0.1	227.5	103	6.50	1.60	6.54	4.94	8.60
6	Póvoa Street, Paredes (Waterfall)	41.195955	-8.328980	6.86	14.84	10.53	196	0.09	248.6	98	5.10	1.66	5.87	4.21	45.78
7	Irivo (before WWTP)	41.189453	-8.336823	7.35	15.04	9.98	185	0.09	233.7	99	5.60	1.83	6.04	4.22	15.33
8	Areias Bridge (after WWTP)	41.170067	-8.348392	7.11	15.47	9.62	201	0.1	237.1	100	5.10	2.08	6.57	4.49	30.77
9	Preisal (before pick up point of underground water)	41.159823	-8.369752	6.90	15.60	10.01	200	0.09	265.4	99	5.00	1.93	6.17	4.25	25.50
10	Alvre	41.133197	-8.418973	7.20	16.19	10.04	184	0.09	256.7	97	1.40	2.02	6.04	4.02	27.38
11	Senhora do Salto (Micro hydric plant)	41.128939	-8.433870	7.14	16.42	10.10	188	0.09	252.6	94	4.90	2.13	5.76	3.63	14.80
12	Covelo (before Ferreira River)	41.105393	-8.472120	6.98	16.80	10.92	190	0.09	263.8	95	4.40	1.48	5.15	3.67	28.36
13	After Ferreira River	41.093909	-8.501425	6.78	17.22	9.87	179	0.08	253.0	89	4.30	2.12	5.82	3.70	40.91
14	Foz do Sousa (Douro influencie)	41.087793	-8.519653	6.99	17.29	9.39	199	0.09	258.1	100	7.00	1.88	5.74	3.86	29.10
15	Douro River	41.088355	-8.520413	6.92	17.33	9.80	195	0.09	255.2	99	7.20	2.27	6.01	3.74	-

Abbreviations: Lat., Latitude; Long., Longitude; DO, Dissolved Oxygen; ORP, Oxidation-Reduction Potential; TDS, Total dissolved solids; DOC, Dissolved Organic Carbon; TC, Total Carbon; IC, Inorganic Carbon.

Table E2. Ions concentration measured in Sousa River (mg L^{-1}).

Target Point	Characteristics / Location	Na^+ (mg L^{-1})	NH_4^+ (mg L^{-1})	K^+ (mg L^{-1})	Ca^{2+} (mg L^{-1})	Mg^{2+} (mg L^{-1})	BrO_3^- (mg L^{-1})	Cl^- (mg L^{-1})	NO_2^- (mg L^{-1})	Br^- (mg L^{-1})	NO_3^- (mg L^{-1})	PO_4^{3-} (mg L^{-1})	SO_4^{2-} (mg L^{-1})
S	Source	-	-	-	-	-	-	-	-	-	-	-	-
1	São Cristovão Street, Lordelo	11.70	n.d.	2.59	8.50	3.37	n.d.	12.79	n.d.	n.d.	20.10	n.d.	8.05
2	Veiga Bridge	11.79	n.d.	2.53	8.55	3.32	n.d.	13.35	0.26	< MQL	19.17	n.d.	8.85
3	Ponte Amial Street, Cernadelo	11.67	n.d.	2.51	8.49	3.32	n.d.	13.38	< MQL	< MQL	19.89	n.d.	8.61
4	Lodares (before WWTP)	11.72	n.d.	2.58	8.21	3.25	n.d.	13.57	< MQL	< MQL	19.87	n.d.	8.48
5	Novelas, Penafiel (after WWTP)	12.52	< MQL	2.85	8.46	3.28	n.d.	14.49	< MQL	< MQL	20.38	n.d.	8.81
6	Póvoa Street, Paredes (Waterfall)	12.10	n.d.	2.67	8.32	3.22	n.d.	14.02	< MQL	< MQL	19.83	n.d.	8.65
7	Irivo (before WWTP)	12.15	n.d.	2.69	8.26	3.23	n.d.	14.09	< MQL	< MQL	19.33	n.d.	8.75
8	Areias Bridge (after WWTP)	13.44	< MQL	2.83	8.01	3.14	n.d.	15.59	0.36	< MQL	17.76	n.d.	9.73
9	Preisal (before pick up point of underground water)	12.98	n.d.	2.76	7.88	3.12	n.d.	15.02	0.30	< MQL	17.67	n.d.	9.41
10	Alvre	12.91	n.d.	2.69	7.70	3.08	n.d.	15.02	< MQL	< MQL	17.65	n.d.	9.38
11	Senhora do Salto (Micro hydric plant)	12.58	n.d.	2.59	7.44	3.01	n.d.	14.74	< MQL	< MQL	17.01	n.d.	9.31
12	Covelo (before Ferreira River)	12.31	n.d.	2.49	7.18	3.02	n.d.	14.56	< MQL	0.33	15.53	n.d.	9.29
13	After Ferreira River	12.14	n.d.	2.67	7.55	3.11	n.d.	14.74	< MQL	< MQL	15.98	n.d.	11.16
14	Foz do Sousa (Douro influencie)	12.43	n.d.	2.63	7.57	3.13	n.d.	14.93	0.25	< MQL	15.75	n.d.	11.29
15	Douro River	12.49	n.d.	2.65	7.62	3.15	n.d.	15.02	0.25	< MQL	15.56	n.d.	11.36

Table E3. Physical-chemical parameters measured in Ave River.

Target Point	Characteristics / Location	Lat. (ºN)	Long (ºW)	pH	Temperature (ºC)	DO (mg L^{-1})	Conductivity (µS cm^{-1})	Salinity (PSU)	ORP (mV)	TDS (ppm)	Turbidity (NTU)	DOC (mg L^{-1})	TC (mg L^{-1})	IC (mg L^{-1})	Flow Rate (m^3 s^{-1})
1.1	Boat Entry	41.578277	-8.130229	5.81	19.31	8.87	89	0.04	187.1	45	0.75	2.63	4.23	1.59	-
1.2	Before small waterfall	41.579992	-8.113193	6.23	13.47	10.32	74	0.03	215.8	96	0.60	1.40	3.01	1.61	-
1.3	River Beach	41.596798	-8.123149	6.38	19.61	8.70	66	0.03	205.1	33	0.50	1.67	3.26	1.59	-
1.4	Natural Zone	41.605776	-8.135009	6.36	19.55	8.58	77	0.03	239.0	38	0.45	1.56	3.41	1.85	-
1.5	Natural Zone	41.595495	-8.147004	6.48	19.77	8.79	74	0.03	245.5	36	0.50	2.90	4.59	1.69	-
1	Source Media	-	-	6.25	18.34	9.05	76	0.03	218.5	50	0.56	2.03	3.70	1.67	-
2	Ponte Men Gutierres - Occasional Discharge	41.576986	-8.166351	6.38	14.15	10.33	72	0.03	266.7	36	0.50	1.93	4.10	2.17	-
3	Standing Water – Forest	41.560289	-8.214434	6.82	15.55	10.18	98	0.04	248.9	48	0.65	2.03	4.95	2.92	-
4	After two tributaries (Pequeno and other)	41.539299	-8.263313	6.93	15.13	10.31	82	0.03	272.4	36	1.80	1.94	4.36	2.42	32.22
5	Before DWTP	41.505453	-8.306600	6.97	15.78	10.83	114	0.05	258.1	56	1.80	1.86	4.18	2.32	45.16
6	After two tributaries (Agrela and das Pontes)	41.471767	-8.346705	7.50	16.82	10.42	104	0.05	218.7	52	2.30	2.21	4.63	2.42	28.01
7	Before WWTP Serzedelo and after Industrial Zone	41.420384	-8.377359	7.48	17.27	10.36	106	0.05	269.8	53	1.30	2.20	4.87	2.67	13.68
8	After tributary and before Vizela River and WWTP Burgães	41.389509	-8.396709	7.66	17.94	10.46	200	0.09	280.8	99	2.30	1.78	7.95	6.18	53.56
9	After WWTP Burgães and Vizela River	41.346214	-8.470378	8.45	17.76	10.81	226	0.11	226.8	113	1.90	2.40	8.14	5.74	54.83
10	Before Este River and WWTP Tougues	41.351377	-8.681185	7.51	19.06	10.30	232	0.11	217.9	116	1.50	3.02	8.97	5.95	34.36
11	Estuary	41.351100	-8.739360	7.06	18.92	9.67	2375	1.23	239.9	1190	3.60	3.71	10.92	7.21	86.67

Abbreviations: Lat., Latitude; Long., Longitude; DO, Dissolved Oxygen; ORP, Oxidation-Reduction Potential; TDS, Total dissolved solids; DOC, Dissolved Organic Carbon; TC, Total Carbon; IC, Inorganic Carbon.

Table E4. Ions concentration measured in Ave River (mg L^{-1}).

Target Point	Characteristics / Location	Na^+ (mg L^{-1})	NH_4^+ (mg L^{-1})	K^+ (mg L^{-1})	Ca^{2+} (mg L^{-1})	Mg^{2+} (mg L^{-1})	BrO_3^- (mg L^{-1})	Cl^- (mg L^{-1})	NO_2^- (mg L^{-1})	Br^- (mg L^{-1})	NO_3^- (mg L^{-1})	PO_4^{3-} (mg L^{-1})	SO_4^{2-} (mg L^{-1})
1.1	Boat Entry	4.53	n.d.	1.00	n.d.	1.13	n.d.	3.93	n.d.	n.d.	2.76	n.d.	1.56
1.2	Before small waterfall	3.39	n.d.	<MQL	n.d.	0.89	n.d.	3.28	n.d.	n.d.	2.58	n.d.	1.05
1.3	River Beach	3.45	<MQL	<MQL	n.d.	0.90	n.d.	3.69	n.d.	n.d.	2.56	n.d.	1.30
1.4	Natural Zone	3.57	<MQL	<MQL	n.d.	0.95	n.d.	3.79	n.d.	n.d.	2.56	n.d.	1.28
1.5	Natural Zone	3.50	<MQL	<MQL	n.d.	0.89	n.d.	3.76	n.d.	n.d.	2.64	n.d.	1.16
1	Source Media	3.69	<MQL	1.00	n.d.	0.95	n.d.	3.69	n.d.	n.d.	2.62	n.d.	1.27
2	Ponte Men Gutierres - Occasional Discharge	4.82	<MQL	1.00	<MQL	1.13	n.d.	4.94	n.d.	n.d.	4.29	n.d.	1.79
3	Standing Water - Forest	6.18	<MQL	1.42	<MQL	1.44	n.d.	6.48	n.d.	n.d.	6.17	n.d.	2.82
4	After two tributaries (Pequeno and other)	5.28	<MQL	1.21	<MQL	<MQL	n.d.	5.53	n.d.	n.d.	5.08	n.d.	2.44
5	Before DWTP	5.21	<MQL	1.23	<MQL	1.28	n.d.	5.53	n.d.	n.d.	5.53	n.d.	2.71
6	After two tributaries (Agrela and das Pontes)	5.88	<MQL	1.46	n.d.	1.43	n.d.	6.34	n.d.	<MQL	6.58	n.d.	3.37
7	Before WWTP Serzedelo and after Industrial Zone	6.32	<MQL	1.44	n.d.	1.48	n.d.	6.52	n.d.	<MQL	6.38	n.d.	3.71
8	After tributary and before Vizela River and WWTP Burgães	23.24	<MQL	<MQL	<MQL	1.78	n.d.	20.38	<MQL	<MQL	7.84	n.d.	7.48
9	After WWTP Burgães and Vizela River	25.81	<MQL	3.19	<MQL	2.02	n.d.	25.37	<MQL	<MQL	9.47	n.d.	9.10
10	Before Este River and WWTP Tougues	25.47	<MQL	<MQL	<MQL	2.35	n.d.	25.53	<MQL	<MQL	11.24	0.42	9.79
11	Estuary	624.18	0.58	30.73	1.66	73.06	n.d.	1048.49	<MQL	3.00	12.93	n.d.	144.94

Appendix F: Concentration of CECs (ng L^{-1}) detected in Sousa and Ave River

Table F1. Concentration of CECs (ng L^{-1}) detected in Sousa and Ave River samples analysed.

Group	Analyte	Sousa River (n = 15)		Ave River (n = 15)	
		Concentration (ng L^{-1})	Frequency	Concentration (ng L^{-1})	Frequency
Anti-inflammatory	Diclofenac	319.83 – 1855.95*	11/15	29.53 – 97.95	10/15
Organic UV Filter	2-Ethylhexyl 4-methoxycinnamate	< MQL – 26.11	14/15	< MQL – 23.86	10/15
Macrolide Antibiotics	Erythromycin	n.d.	n.d.	5.62 – 9.30	5/15
	Clarithromycin	3.88 – 5.91	15/15	3.81 – 4.28	7/15
	Azithromycin	2.42 – 4.83	11/15	1.91 – 3.73	11/15
Pesticide	Methiocarb	n.d.	n.d.	n.d.	n.d.
Neonicotinoids	Imidacloprid	n.d.	n.d.	< MQL – 136.52	9/15
	Thiacloprid	n.d.	n.d.	n.d.	n.d.
	Thiamethoxam	n.d.	n.d.	< MQL – 88.34	7/15
	Clothianidin	n.d.	n.d.	n.d.	n.d.
	Acetamiprid	n.d.	n.d.	n.d.	n.d.

Abbreviations: n.d., not detected; MQL, Method Quantification Limit; *, After dilution.